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Studies in Iron Chlorosis of Leaves

Narayan Gunderao Perur

STUDIES IN IRON CHLOROSIS OF LEAVES

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Narayan Gunderao Perur

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N. G. Perur

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INTRODUCTION

Normal healthy growth and development of plants is controlled by certain factors known as factors of plant growth. They are soil, climate, and plant inheritance. The soil contains many nutrient elements needed by plants both in macro and micro quantities. Iron is one of the essential elements required by plants in small amounts.

When plants are starving for any of the essential nutrients, characteristic symptoms usually appear as certain abnormalities, discoloration and deformation of leaves, fruits, and other parts. The deficiency symptoms, though not easily recognizable in some cases at the beginning, are quite apparent in advanced stages of plant growth.

The characteristic symptom of iron deficiency in plant nutrition is chlorosis. Chlorosis is a general term which denotes a lack of chlorophyll in leaves and replacement of the normal green color either wholly or in part by yellow color. In most cases the chlorosis is characterized by a distribution of the yellow color throughout the green in patterns. The more specific term, iron chlorosis, refers to chlorosis due to iron deficiency in the plant and which can be alleviated by providing the plant with suitable iron compounds.

The first symptom of iron deficiency is the yellowing of younger leaves in the areas between the veins while the veins remain green. In severe conditions of iron deficiency the entire leaf may be yellow or even white. Plants suffering from a lack of iron, besides being chlorotic, are spindly and weak and will fail to produce a normal set of

bloom and fruit. Growth is retarded and plants may eventually die. Iron is comparatively immobile in plants. When a deficiency sets in, leaves once green remain so, while the younger leaves become chlorotic.

Under soil conditions more than one factor may be involved in the development of chlorosis in plants. The soil may have sufficient iron but certain conditions, such as high pH, excess water, excess lime and phosphorus, may tie up the amount for normal growth. The deficiency of magnesium, manganese, and zinc and infection by certain viruses also bring about chlorosis. A correct diagnosis of the chlorosis problem is thus complicated especially for plants growing in soil.

Plant species differ in their requirement for iron and its absorption. Some plants grow well where others will not grow without developing iron chlorosis. Even the varieties of the same species differ widely in their susceptibility to iron chlorosis. PI soybean PI-54619-5-1 (Glycine max) has been found to be more easily susceptible to chlorosis than Hawkeye soybean. It appears that the availability of any supply of iron in the growing medium to plants will depend upon both the plant species as well as the growth medium. It would therefore be of importance to determine why different plants grown under similar environment differ in their susceptibility to chlorosis.

It may be a paradoxical statement to say that the natural growth medium of land plants, the soil, is least adapted for the study of the indispensable nature of plant nutrients. It was with the artificial nutrient media, water and sand cultures, that the essential status of the various elements found in plants and derived from the soil was established. The artificial water culture technique is therefore a powerful and discriminating tool in studying the role of inorganic

nutrients in plant nutrition. The technique enables one to isolate an individual factor and study its effects, independent of others.

Iron as an essential nutrient element for plants and its importance in chlorophyll production has long been known. However, its exact role in the synthesis of chlorophyll is not known. Aside from its connection with chlorophyll, its other manyfold functions in plants are listed below. Iron is taken up by plants in small amounts. It can therefore be suggested that it might mainly function as a catalyst in various activities of plant growth and maturity. Iron is also used by plants directly as a constituent of cell wall tissue. It is found to affect oxidation and reduction processes in the plant. Manganese and iron form a pair of mutually co-ordinating catalysts for oxidation and reduction processes involving the addition and removal of oxygen. Iron in leaves also occurs in combination with some proteinaceous fractions, presumably enzymes, which activate formation of certain other proteins intimately related to chlorophyll.

All known enzymatic systems depending on iron involve porphyrin molecules. Catalase, peroxidase, cytochrome c and chtochrome oxidase activities have been linked up with this element in some way. Other enzymes like polyphenol oxidase and succinic dehydrogenase have also been known to be interrelated in effect with iron. Certain respiratory enzymes required for salt accumulation are also mediated by iron enzymes. In all these reactions brought about by such enzymes, iron plays a vital part. Its role in chlorophyll production and its relation to other metals is of great significance in the physiology of plants.

When iron is incorporated into the porphyrin ring, the catalase and peroxidase activities are enhanced. When the iron prophins become attached to specific proteins not only do these particular protein

compounds acquire a marked degree of specialization and enormous enhancement of activity but they are also stabilized and protected. Depending upon the protein to which the iron porphin is attached, it may function as a transporter of molecular oxygen, transporter of electrons, and activator of oxygen. Porphyrins by themselves show no catalytic activities. It is only when the porphyrins form co-ordination compounds with iron that they appear to develop properties of great physiological significance.

The iron itself does not enter into the composition of chlorophyll molecules. But normal green color of leaves is not produced in the absence of iron. Although it may safely be concluded that iron in involved in the problem of chlorosis of leaves, evidences are insufficient to ascribe the chlorosis to the total iron content of such leaves. The absorbed iron in chlorotic leaves seems to be not in a state readily utilizable by the leaf enzymes for the synthesis of chlorophyll.

The importance of enzymes in the physiology of plants is being appreciated more and more as a wide spectrum of enzymes have been purified, characterized, and their biochemistry studied in detail. Many of these enzymes which mediate important metabolic processes of the cell are present in the soluble protein fraction of the cytoplasm. Previous to 10 to 20 years, bulk analysis of plant material for organic and inorganic constituents was commonly done to study the physiological changes in plants. Today a galaxy of physical and chemical techniques are available to isolate and study individual components of cell tissue.

With the aid of low temperature techniques, efficient buffering systems, colloidal mills, ultra centrifugation, electrophoresis,

chromatography, and electron microscopy, plant scientists have now started fractionating and studying the cell constituents. More than any other tool, the ultra centrifuge and electrophoresis have made it possible to separate individual proteins from complex mixtures such as the proteins of cytoplasm of green leaves.

In recent years more interest is seen in the study of cytoplasmic proteins. Numerous electrophoresis studies of serum proteins have been made in medical science to characterize various pathological conditions (Eggman, 1953). Electrophoresises of virus proteins in leaves have also been reported (Wildman and Jagandorf, 1952). Similar studies of physiologically diseased leaves may yield fruitful information.

One of the happiest aspects of atomic discoveries is the peacetime application of atomic energy. The use of radioactive isotopes in plant science is one of its applications. Radioactive isotopes are used as tracers to study the absorption, translocation, and utilization of nutrients by plants. Investigation of a whole range of phenomena in biochemistry and physiology which otherwise would not have been subjected to direct experimentation is made possible by tracer techniques. Besides, measurement of radioactivity may be more sensitive than many chemical methods. Getting an exact measure of the nutrient uptake by plants had been one of the toughest problems until now. Radioactive isotopes contribute to the solution of these problems. Absorption and translocation patterns of both macro and micro nutrient elements in plants have been studied by the use of the tracer technique.

Even with the advent of all these techniques, the study of plant proteins has lagged behind the similar studies of animal proteins. This has been due in part to the relative ease with which pure proteins

such as serum albumins may be secured for study in animals and in part to its possible medical application.

Use of the modern tools of research may be of great help in the study of the problem of iron chlorosis. Changes in mineral composition, chlorophylls and carotenoid pigments of a leaf, effect on enzyme activities, localization of iron, and pattern of protein distribution within the leaf cell tissues were some of the aspects of the chlorosis problem proposed for investigation. An account of the approach to these intricacies and the results obtained are discussed in the following pages.

REVIEW OF LITERATURE

That iron is an essential element has been known for decades. Gris (1844) first reported the use of soluble iron compounds for alleviating, at least temporarily, the chlorosis of leaves. Since then, many investigations have been carried out all over the world and at present there is a voluminous literature on different aspects of the chlorosis problem. The present review covers only the general aspect of chlorosis and its effect on chemical composition, respiration, photosynthesis, enzyme activities, leaf proteins, and methods used in such studies.

It is recognized that some plants grow well where others cannot grow without producing chlorosis. This has been said to be due to the difference among plant species. Very little work seems to have been done to explain such a difference.

Varietal Differences

Mazé (1913) first observed that plant species differed widely in their susceptibility to chlorosis. While lupine and vetch became chlorotic on high lime soil, corn did not show iron deficiency symptoms. Weiss (1943) showed that the difference in efficiency of iron utilization between the chlorosis susceptible and nonsusceptible soybeans was conditioned by a recessive gene. Gericke (1940) reported that the structure of root systems was associated with the susceptibility or otherwise of plants to iron chlorosis. Monocotyledons by virtue of their more fibrous root system were efficient in utilizing the iron from the growth medium and thus were less susceptible than the dicotyledons.

Brown (1953) conducted studies by growing several plant species on calcareous and organic soils in order to test their susceptibility to iron chlorosis and copper deficiency. He noticed that those plants which did not develop copper deficiency symptoms when grown on an organic soil poor in copper could exhibit iron deficiency chlorosis on calcareous soil. A good example of varietal difference noticed in Brown's experiment was that of two soybean varieties. While Hawkeye soybean could grow without chlorosis on a calcareous Millville soil, PI soybean produced chlorosis. Probably PI variety failed to absorb sufficient iron from the calcareous soil. These observations were further confirmed by autoradiographs (Brown and Holmes, 1955).

Holmes and Brown (1955) found the iron requirement of the two soybean varieties to differ when grown in solution cultures at different concentrations of iron. The chlorosis susceptible variety required a higher iron concentration to produce green plants than the nonsusceptible variety.

Another interesting manifestation of a varietal difference in susceptibility to iron chlorosis is the work of Brown et al. (1959). Using a split root technique, they demonstrated an internal inactivation of iron in PI soybean, principally from the combined effects of phosphorus and calcium, both of which are supposed to induce chlorosis when present in excess. The Hawkeye variety remained green through conditions under which PI variety became chlorotic. This indicated a difference in the capacity of these two soybean varieties to absorb and retain iron mobile in an environment conducive to inactivation. The authors postulated the quantity or the quality of natural iron chelation as the factor responsible for such difference in susceptibility to

chlorosis among varieties.

Soil Conditions

Apart from simple iron deficiency as the main cause of chlorosis, there are many factors both direct and indirect which induce chlorosis. Iron, manganese, and phosphorus concentrations in the soil have been found to influence chlorosis. Johnson (1917) found that in Hawaiian soils containing high manganese, plants affected by chlorosis were remedied by applying soluble iron compounds. In his studies it was observed that the manganese to iron ratio and not the total amount of either element present was important in causing chlorosis. These findings are in agreement with those reported by Shive (1941), and Olson (1950). Somers and Shive (1942) postulate that high soluble manganese to iron ratio in plant tissues may cause the oxidation of ferrous to ferric iron, resulting in the precipitation of iron in the form of ferric organic complexes. Bennett (1945) on the contrary, experimenting with tomato plants (which are relatively resistant to iron chlorosis) found that manganese neither acted toxic nor interfered with the utilization of iron in the leaf. He contended that the manganese to iron ratio had no relation to the chlorosis produced.

Iron chlorosis has been noticed not only on alkaline soils but also on acid soils. In their extensive survey of Florida's citrus growing area, Leonard and Stewart (1952) recorded that three-fourths of the area was acidic and that iron chlorosis was widely distributed. Reuther and Smith (1952) and Smith and Specht (1952, 1953) from their field and greenhouse studies came to the conclusion that the acid soil chlorosis of citrus plants in Florida, in many instances, was caused by a high concentration of heavy metals in relation to the available iron content of the soil.

The most widespread incidence of iron deficiency in plants occurs on alkaline calcareous soils. Chlorosis of plants under this condition is commonly termed as "lime-induced chlorosis." Thorne et al. (1950) have surveyed the existing facts concerning the cause of lime-induced chlorosis and have discussed the following hypotheses: (a) High pH and excess quantities of lime make soil iron less available to plants. (b) High soil moisture, poor aeration, and cool temperature disturb plant metabolism to an extent that iron is inactivated (Burtch et al., 1948). (c) Phosphorus may precipitate iron either in the soil or in plant tissues (Biddulph, 1947). (d) High manganese in soils or plants may oxidize iron to an inactive state (Somers and Shive, 1942). (e) An excessive amount of potassium in leaves may displace iron from an active enzyme surface and thereby disrupt metabolic processes (Kirsanov et al., 1937). (f) An unbalanced cation content of plant tissue may lead to disrupted synthetic activities with an abnormal accumulation of certain organic acids and an increase in soluble nitrogen at the expense of insoluble protein (Bennett, 1945). (g) Soil and plant conditions associated with excess lime in soil favor the oxidation of iron to the less active ferric state and its fixation in compounds of low biological activity (Bennett, 1945; Oserkowsky, 1933; Thorne and Wallace, 1944). (h) None of these hypotheses unify the observed facts into a rational concept of the disease.

Leaf Analysis

There is a wide controversy in the literature regarding the composition of leaves affected by chlorosis. If chlorosis is considered as an iron deficiency then chlorotic leaves should, in comparison with healthy ones, contain less iron. However, many workers have reported findings contrary to this effect. Several experiments have been

reported in which chlorotic leaves when analyzed were found to contain a higher iron concentration than the normal green leaves (Oserkowsky, 1933; Iljin, 1952; Warnock, 1952; DeKock, and Morrison, 1958). This suggests that much of the iron present in chlorotic leaves is in an insoluble form and is not effective in the metabolic processes. Investigations of iron unavailability has led to general acceptance of the theory that it is the soluble form in the plant which plays the important role in metabolism. It is evident that iron may become inactivated and therefore made unavailable for the vital processes of the plant (Somers and Shive, 1942). This theory is also supported by Bennett (1945) who postulates that chlorosis is due to inactivation of iron in leaves. However, contrary to the belief of Somers and Shive (1942), Bennett (1945) says that inactivation of iron in the leaf is not due to its precipitation at the higher pH of sap. The reason, he says, is that in many cases chlorotic and green leaves have the same pH. Other workers suggest that inactivation of iron is associated with an excess of phosphorus, potassium, and manganese.

Oserkowsky (1933) reported that chlorotic pear leaves might contain as much or more iron than green leaves of the same age taken from the same tree regardless of whether the iron content was expressed on the fresh weight or the dry weight basis. He introduced the term "active" for that part of the total iron in leaves which could be extracted with one normal hydrochloric acid. He noticed that this active iron was correlated with chlorophyll content. This view is also held by Thorne and Wallace (1944), Bennett (1945), Jacobson (1945), and McGeorge (1949) by virtue of their similar findings. Bennett (1945) found no direct relation when the active iron was

expressed on the dry weight basis but suggested that since active iron is bound to enzyme systems whose activities are functions of the protein fraction, the latter constituent should serve as the basis for evaluating this form of iron in leaf tissue.

Jacobson (1945) fractionated the chloroplast bodies of leaves and found that the acid-soluble iron content was closely related to chlorophyll content. Lindner and Harley (1944) found that when iron removed by half normal hydrochloric acid was low, iron deficiency symptoms were severe. They noticed no direct relation between chlorophyll concentration and either half or one normal hydrochloric acid-soluble iron. McGeorge (1949) analyzed green and chlorotic leaves from a number of orchard and field crops in Arizona to determine whether any correlation existed between citric and oxalic acids and active iron in lime-induced chlorosis. His observations were that chlorotic leaves contained less active iron than did green leaves. In his previous experiments he had found that iron uptake for seedlings grown on soils that produced chlorotic plants was greater than for the normal green plants. Olson (1950) did not find significant differences in the active iron content of chlorotic and non chlorotic leaves. His test plants were sorghum. Smith et al. (1950) found an inverse relationship between the total iron of citrus leaves cleaned with detergent and the degree of iron chlorosis. The chlorotic leaves also had an abnormally low manganese content.

Iljin (1951) postulated the concept that deficiency in the formation of chlorophyll pigment in chlorotic leaves is just a single result among others, all of which combine in reflecting a crucial cause for lime-induced chlorosis. He did leaf analysis of several plant species and noticed, in certain cases, higher iron concentration in chlorotic

leaves than in non-chlorotic ones. Iljin (1952) in another study found that iron content of the sap of chlorotic leaves exceeded that of normal plants. Warnock (1952) also observed that the iron concentration of expressed sap of green and chlorotic leaves was about the same.

The anomoly noticed in the iron content of chlorotic plants may be due to defective sampling of plant material and surface contamination. The age of the plant is an important factor for consideration while sampling. Generally, young tissue contains a higher percentage of mineral matter. Unless the plant parts of similar physiological growth are sampled for analysis, the results will not be comparable. Many of the abnormal figures reported for deciduous iron in chlorotic leaf samples collected from deciduous trees and field crops might have been subjected to this discrepancy.

The surface contamination is another source of discrepancy. Unless care is taken to remove carefully all the surface contamination, results for iron content are invariably higher. Therefore, if the age of the leaves sampled and the removal of surface contamination are thoroughly considered in determining iron in plant material, it might be possible to arrive at a better agreement in the literature on the iron content of chlorotic and nonchlorotic plant tissues. Wallihan (1955) considered this issue carefully and made an emphatic statement that iron chlorosis of citrus plants is caused by simple iron deficiency in leaves and that chemical analysis can be a useful tool in studies of iron nutrition of citrus plants provided contamination of leaves is avoided. Jacobson and Oertli (1956), in an effort to establish a relation between iron and chlorophyll contents in chlorotic sunflower leaves, came to the conclusion that chlorosis is initially caused by a

simple deficiency of iron but under some conditions subsequent correlation between iron and chlorophyll contents may be obscured because of effects related to the deficiency.

In many examples quoted above, the leaf analysis could not show a direct relationship between chlorophyll and iron. However in isolated chloroplasts, according to Seibic (1911), as much as 82 percent of the leaf iron is present in the chloroplasts. Smythe and Schmidt (1930) studied the mode of combination of iron with nitrogenous compounds and observed that in green leaves a considerably larger amount of nitrogen was present as protein nitrogen than in the case of chlorotic leaves. This they attributed either to the direct relation between iron and protein synthesis or to a decrease in protein synthesis brought about by a reduced rate of photosynthesis in chlorotic leaves. They also found a good correlation between iron and protein content as well as between iron and chlorophyll contents. They contended that since half the leaf protein was present in chloroplasts, large differences in protein composition of the two type of leaves involved the chloroplasts. They suggested that the chlorosis was characterized by an imperfect development of the chloroplasts caused by lack of iron salts. In tobacco leaves the active iron (soluble in one normal hydrochloric acid) is localized solely in the chloroplasts, but other fractions, both acid-soluble and acid-insoluble iron, are also present (Jacobson, 1945).

The effect of chlorosis on the composition of other related elements as well as on organic constituents of leaves is also worthy of consideration. Many have reported on abnormal accumulation of mineral elements and organic compounds in chlorotic plants. Lindner and Harley (1944) found more potassium and less calcium in leaves affected with

lime-induced chlorosis. They postulated that a high potassium level in leaves would probably replace the iron from the enzyme responsible for chlorophyll formation. This inactivated the enzyme, resulting in chlorosis. Thorne and Wallace (1944) in their analysis of chlorotic leaves from peach, pear, grape, prune, and apple plants observed more potassium and nitrogen but less iron and calcium in chlorotic leaves than in green ones. Thorne et al. (1950) suggested that the upset of potassium to calcium ratio was the result of chlorosis rather than a cause of it. Olson (1950), contrary to one's belief, reported less magnesium in green leaves of sorghum compared to the chlorotic ones. He did not consider calcium as being related to chlorosis.

McGeorge (1940) noticed excess accumulation of potassium and organic acids in chlorotic leaves. His results have now been confirmed by the findings of Iljin (1951, 1952) and Wadleigh and Brown (1952). Iljin describes the nutritional status of chlorotic leaves as the accumulation of potassium and calcium in addition to a fluctuating level of phosphorus coupled with excessive soluble nitrogen, reducing sugars and organic acids, especially citric acid. Larger amounts of unidentified acids in the most severely chlorotic leaves were also noticed by him. This has been reestablished recently by Rhodes et al. (1959) in their studies on lime-induced chlorosis with bush bean leaves using C^{14} as a tracer element.

Often elemental ratios of potassium to calcium and phosphorus to iron have been found upset in chlorotic leaves. Lindner and Harley (1944) reported a higher potassium to calcium ratio on chlorotic leaves. DeKock (1955) related the ratio of phosphorus to iron with the extent of chlorosis severity. Chlorotic leaves had a higher ratio. He

reported a P:Fe ratio of 60 to 70 for chlorotic leaves and 40 to 50 for normal green leaves.

Whether the reduction in chlorophyll content of leaves as a result of chlorosis simultaneously affects other plastid pigments like carotenoids is not well known. Mackinney (1935) reported that, in general, chlorophyll and carotenoids fluctuated together though the carotene to xanthophyll ratio was lower in chlorotic leaves. Sideris et al. (1956) studied pineapple chlorosis in relation to iron and nitrogen. They found that iron deficiency caused a decrease in chlorophyll, carotenoids, and protein contents of chlorotic leaves.

When the deficiency of a nutrient sets in, a reduction in the growth of the plant is generally manifested which consequently affects yield or weight of the plant. However, Pound and Nelkie (1950) have recorded increased wet and dry weight of leaf tissue in iron deficient plants of tobacco. Their observations were on unit area basis. However, percent dry matter was about the same at all the levels of iron. Nelkie and Pound (1958) also found similar results in manganese deficient leaves of the above mentioned plant. In both cases, the authors believe this increase was due to an increase in leaf thickness.

Importance of reaction or the pH in biological systems need not be over emphasized. Normally, most of the biological fluids are highly buffered and wide fluctuations in pH may not be expected. Protoplasm in the cell contains a mixture of many organic and inorganic compounds. If these are to be utilized during the metabolic processes, they should be held in soluble or readily available form. This is made possibly by maintaining a fine balance of pH. When this cannot be achieved due to a lack of one or more nutrient elements, to physiological diseases, or

to unfavorable environments, abnormalities in the physiology of plants may be expected. Since, as mentioned earlier, the plant sap is sufficiently buffered ordinarily, slight deficiencies or other adverse conditions should have only a slight effect on its pH. Apart from this theoretical consideration, perhaps, due to the different conditons under which the experiments were conducted, diverse reports on this subject are met with in the literature.

Several investigators have shown the pH of the growth medium to have a profound influence on the pH of plant sap. Ingalls and Shive (1931) studied the relation of hydrogen ion concentration of tissue fluids to the distribution of iron in plants. They noticed that the soluble iron content of plants varied directly with the hydrogen ion concentration. Plants in which the tissue fluids had a low pH showed high total but relatively low soluble iron content, and those in which the tissue fluids had high hydrogen ion concentration values showed low total iron but relatively high soluble iron content. Oserkowsky (1932) noticed no significant difference between hydrogen ion concentration and iron content in tracheal sap of green and chlorotic branches from pear trees in the same orchard. However, Rogers and Shive (1932) found a direct correlation between the pH of the tissue fluids and the amount of total and soluble iron present in the tissue. In general, as the pH increased, the total iron content increased and the soluble iron decreased.

Hurd-Karrer (1939) reported that the pH values of the expressed juice of leaves of normal wheat plants during their vegetative period of growth ranged from 5.85 to 6.30 but were usually between 5.9 and 6.2. However, during the maturation period the acidity increased, the pH value falling to about 5.5. She also noticed that unhealthy plants

became prematurely acid but none reached pH values below 5.5. She did not find significant varietal difference in the pH values for plants grown under conditions equally favorable for normal growth. She also found that liming the soil had no effect on the plant juice acidity in all the experiments. Finally, in compilation of reports from the literature for other crops, the author noted that the majority of plants had a pH between 5.5 and 6.5. Arnon and Johnson (1942) also reported that pH of the nutrient solution had a significant effect on the pH of the expressed sap. Sideris and Young (1944) concluded that the high acidity values of the sap from the chlorophyllous sections of the leaves and the exceedingly small amounts of iron present in the same tissues precluded the possibility of iron precipitation in the leaf tissues.

The above reported fact of Hurd-Karrer (1939) does not agree with that of Small (1946) who states that in general the pH of plant sap is raised by liming the soil. In support of this statement the work of Baxter and Belcher (1955) indicated that the root sap of orange trees growing on alkaline soil had a lower hydrogen ion concentration than the sap from tree roots growing on acid soil.

The oxidation-reduction potential of a chemical system is a measure of the tendency for oxidation or reduction to occur. Hewitt (1951) considered the importance of oxidation-reduction potential in determination of the relative activity of several metals, especially iron and manganese, in inducing chlorosis. Bennett suggested that iron directly associated with chlorophyll was ferrous iron, whereas residual iron was ferric ion; and the distribution between these two forms was undoubtedly influenced by the oxidation-reduction potential in the cell.

Photosynthesis and Respiration

Importance of photosynthesis in green plants is well known. Apart from the synthesis of carbohydrates, which is the main source of energy in respiration, photosynthesis has a pronounced effect on cation absorption (Hoagland and Broyer, 1936). The conversion of neutral substances, such as carbon dioxide and water, into a number of ionized metabolites, organic acids, phosphorylated compounds (Calvin and Bensen, 1949) creates a demand not only for cations but also for particular anions such as phosphate and nitrate.

Photosynthesis has been shown to affect the assimilation of nitrate (Burstrom, 1953; 1945) and the carbohydrate status of roots (Hoagland and Broyer, 1936), of which the latter may govern the absorption of ions by root cells. For all the vital processes taking place inside the plant body, energy is to be expended, the source of which is the carbohydrate which in its turn is the product of photosynthesis. Carbohydrates are one of the important substances to start with for the synthesis of other complex organic compounds like fats and proteins. Reduced photosynthetic activity in chlorophyll deficient leaves is naturally expected. Fleischer (1935) has correlated photosynthesis with the degree of chlorosis or the amount of chlorophyll present in plants supplied with low levels of iron.

Respiration is one of the characteristics of all living bodies. Aerobic respiration involves absorption of oxygen and evolution of carbon dioxide with simultaneous liberation of energy. This energy is utilized in the absorption of nutrients and the synthesis of complex organic compounds. It has now been clearly established that entry of salt into plant cells is a process requiring energy. Respiration in

plants is affected by such factors as temperature, food supply, tissue condition, light, water in the tissue, and oxygen concentration. Glenister (1944) correlated respiration with the degree of chlorosis or the amount of chlorophyll present in sunflower plants deficient in iron. Hill et al. (1959) conducted respiration studies on effects of atmospheric fluorides and various types of injuries on the respiration of leaf tissue. They used seven species of plants. Although the authors did not notice any direct effect of the fluoride treatment on respiration, an increase in oxygen uptake related directly to the development of leaf necrosis was found in varieties of gladioli. Injuries also induced an increase in respiration rate.

Enzyme Activity

Enzymes are indispensable compounds which play a key role in metabolism by bringing direction and control to the physiological processes of living cells. It is the enzyme systems of cells which determine the kinds of chemical reactions that can occur (Meyer and Anderson, 1952). Any change in the enzyme complement of living cells is immediately reflected in a change in the physiological processes of the cell (Beadle, 1948). Bennett (1945) recommended that since active iron is bound to enzyme systems whose activities are functions of the protein fraction the latter constituent should serve as the basis to evaluate this form of iron in the tissues. Arnon (1951) considered the approach of estimating the activity of enzyme systems controlled by iron to evaluate chlorosis as the pertinent one to follow.

Hill and Scarisbrick (1951) discovered a new cytochrome component present only in photosynthetic tissues which they named cytochrome f. Hill and Hartree (1953) has made the suggestion that cytochrome f may

play a role in photosynthesis because of its general presence in the photosynthetic tissues and its localization with the chloroplasts. Brown and Hendricks (1952) postulated that when a nutrient is limiting in the nutrition of a plant the deficiency will be indicated by a reduced activity of an enzyme requiring this element. They grew various plants on organic and calcareous soils and found that catalase activity was low when iron supply was limited in corn, tobacco, lupines, and several other crops. Ascorbic acid oxidase activity was found markedly reduced by a limited copper supply and was correlated with a reduction in yield in wheat which has a copper terminal oxidase. Appleman (1952) while studying catalase-chlorophyll relationship in barley seedlings noticed that the catalase activity was higher in etiolated seedlings than in green ones. Barley plants in their early stages of magnesium deficiency had a higher catalase activity than similar plants grown in a complete culture solution. Catalase of etiolated seedlings had a lower activation energy than the catalase in the green seedlings. Further, the author postulated that there is a dynamic equilibrium between the porphyrin-proteins in the chloroplasts. When rapid chlorophyll synthesis takes place, catalase activity suffers a decrease. When chlorophyll synthesis is blocked, catalase activity rises rapidly, if it is at a low level, or it does not decrease if it is at a high level.

Brown (1953) noted that copper-requiring systems (ascorbic acid oxidase) seem to predominate in most plants not susceptible to chlorosis. Catalase has often been implicated as the "oxygen liberating" enzyme of photosynthesis. Mahler and Elowe (1953) pointed out that in absence of iron, the reduction of cytochrome c was greatly inhibited. Weinstein and Robbins (1955) studied the effect of different iron and manganese

nutrient levels on the catalase and cytochrome activity of green and albino sunflower leaf tissue. Their observations were that low levels of catalase and cytochrome oxidase activities were found in both green and albino leaf tissues of plants grown with low nutrient levels of iron or high nutrient levels of manganese.

Since the property of turning red in active metabolizing tissue was first reported by Kuhn and Jerchel (1941), 2,3,5-triphenyltetrazolium chloride (hereafter abbreviated as TTC) has been extensively employed as an indicator of high metabolic activity in a number of plant and animal tissues. Brown (1954) observed that TTC was much more readily reduced in corn containing sufficient copper than in those deficient in the element. This indicated a possible change in the effectiveness of the reducing or oxidizing enzymes. Such a condition could be expected to affect the valency of iron and its absorption and transport in the plant.

Fractionation of Leaf Proteins

During the last few years many papers have been published on plant proteins. Interest in the biochemistry and metabolism of proteins in plants is increasing because of their association with enzymes and the fundamental role these molecules play in most biological phenomena. the leaf is a seat of enzyme activity. Leaf proteins are undoubtedly involved for a large part of the photosynthetic reactions.

A leaf is made of heterogenous substances. The separation of a single component in pure form, therefore, invites many problems. In all plant tissues, the location of protein relative to the architecture of the cell presents extraction problems. Apart from the interest in the physical, chemical, and enzymic properties of cytoplasmic proteins, leaf proteins have been subjected to studies in their relation with the

localization of enzymes and metals in different particulate matter inside the cell.

Stafford (1951) did an interesting study of intracellular localization of enzymes in pea seedlings. She used distilled water as the medium for grinding while homogenizing in a Waring blendor. The various fractions were then separated by differential centrifugation. While comparing the different extracting mediums, she found that phosphate buffer offered no advantage over distilled water, and isotonic or hypertonic potassium chloride or sucrose solutions inhibited some of the enzymes studied. Maximum cytochrome activity was found on particles isolated at 16500 X g. McClendon (1952) in his studies on the intracellular localization of enzymes in tobacco leaves used 0.5 M sucrose and 0.01 M phosphate buffer at pH 6.8 for homogenizing. The leaf material was homogenized in a blendor run for 30 seconds at full speed. He noticed that longer times or slower speeds were not advantageous for recovery of high yields of whole chloroplasts. Homogenate was further fractionated by differential centrifugation. In another investigation of cytoplasmic proteins of green leaves, the author (McClendon, 1953) found that cytochrome oxidase was mainly associated with the mitochondria while catalase was present in all fractions in quantities roughly proportional to the protein content and was not exclusively with the chloroplasts.

Eggman (1953) suspended his leaf samples in 0.5 M malate buffer of pH 7.0 and used Epinbock colloid mill for grinding the tissue. He studied the cytoplasmic proteins of green leaves and noted that the soluble cytoplasmic proteins of leaves of a number of dicotyledonous plant species in electrophoresis migrated as a single major component and from one to six minor components, the number depending upon the

species. James and Das (1957) employed 0.067 M phosphate buffer of pH 7.3 in 0.3 M sucrose solution for isolating chloroplasts and mitochondria. Here also a blendor was used for homogenizing but it was run at full speed (11000 rpm.) for three consecutive periods of 10 seconds with short breaks, to prevent rise of temperature. Using a density gradient medium of sucrose and diodene, they could isolate chloroplasts and mitochondria without much contamination from other particulate bodies.

Gordon (1958) achieved the separation of intracellular organelles by grinding seedlings of the mung bean plant in a chilled mortar with an equal volume of 0.2 M KH_2PO_4 - Na_2HPO_4 buffer in 0.3 M sucrose solution (pH 7.0) and centrifuging at different speeds. The same procedure was employed by Skok et al. (1958) in their investigation of distribution of boron in cells of dicotyledonous plants in relation to growth.

METHODS OF PROCEDURE

Scope of Present Investigation

From the above review it is seen that the study of iron chlorosis has been reported by several workers. However, there does not seem to have been complete agreement even on a single item. The factors governing chlorosis are many and complex. Again, the results of experiments conducted in the field are difficult to compare. Aside from the soil factors, plant species, age of the plant, leaves sampled, and the methods employed were not all the same. The scope of the unsolved problems in iron chlorosis is therefore considerably broader and it was thought that isolating a single factor and studying its effect would help in better understanding the situation and interpreting the results. With this end in view the investigation reported in the following pages was planned.

Experiments were conducted by growing plants in solution culture under more or less controlled conditions of nutrient levels, light intensity, and temperature. Simple iron deficiency was induced by withholding the iron supply. Leaf samples were analyzed for some inorganic constituents, protein, and enzyme activities. An attempt was also made to fractionate the leaf proteins and determine the amount of iron associated with each fraction. In a few cases radioactive iron (Fe^{59}) was used for tracing the element in the protein fractions. Investigation was carried out on two plant species in order to guage different responses for iron deficiency.

Materials

Corn and soybean were used as test plants in the experiments. Corn seeds, variety P.A.G.-444 (Pfister Associated Growers, Inc.), harvested in the previous season, was secured from Professor Rex Nielson of the Agronomy Department, Utah State University, Logan; pure line bred soybean seeds of the Hawkeye variety were obtained from Dr. G. W. Miller of the Botany Department, Utah State University, Logan.

For all the analytical work only the analytical reagent grade chemicals were used. Even the stock solutions for the culture solutions were prepared from pure chemicals. Anticipated amounts of the chemicals needed for the whole period of work were secured beforehand and stored in a cool dry place. This ensured constancy of the reagent quality throughout the work. Except for the sulphuric acid, no attempt was made to further purify the chemicals. All the prepared reagents were stored in clean dustproof stoppered bottles or they were covered with beakers to avoid any contamination from dust. Some of the reagents such as buffer solutions, hydrogen peroxide, and other assay chemicals which are both temperature and light sensitive, were stored in a refrigerator. The cytochrome c preparation (obtained from Sigma Chemical Company, St. Louis, Missouri) required for cytochrome oxidase enzyme activity was stored in a vacuum desiccator in a refrigerator. The Fe^{59} needed for tracer work was obtained from the Isotope Division of the U.S.A.E.C., Oak Ridge, Tennessee.

For all the analytical work only glass-redistilled water was employed. Operations, including preparation of reagents, rinsing of apparatus, dilution, and extraction, were all done in glass-redistilled water. However, for culture solutions deionized water was used. The ordinary laboratory distilled water was allowed to pass through the

resin columns. Before use, the deionized water was stored in a polyethylene tank so that the dissolved carbon dioxide could come to equilibrium with the atmospheric carbon dioxide. Such water had a pH of 8.2 and conductivity less than 10×10^{-5} mhos/cm.

The cold room of the Botany Department, Utah State University, was used for such operations as plant sampling, quick weighing on torsion balance, enzyme preparations, and fractionation of leaf proteins by differential centrifugation. The temperature fluctuation of the cold room was from 30° to 38° F.

Any special instrument used for the analytical and assay work is described under respective methods.

Methods of Analysis

Moisture

An aliquot portion of the plant sample (about one gram) was weighed on a chemical balance and heated in an electric oven at 105° C. for 36 hours. The oven was equipped with an air-draft switch. After cooling to room temperature in a dessicator, the sample was weighed again. From the difference in the two weights, percent moisture and percent dry matter were calculated.

Total nitrogen

The method employed was essentially the same as the one recommended in A.O.A.C. methods of analysis (1945) with modifications, if any, to suit the sample conditions under test.

About 1.0 g. of the green leaf material or 0.1 g. of the dry sample was weighed on a chemical balance and transferred to a kjeldahl flask. About 30 ml. of concentrated sulphuric acid containing one percent salicylic acid was added along with five grams of sodium thiosulphate

one teaspoon of the sodium sulphate-catalyst mixture ($CuSO_4 \cdot 7H_2O$ + HgO + Se + Na_2SO_4). The contents of the flask were heated slowly at the start with occasional swirling round till vigorous action of sodium thiosulphate ceased. The temperature was then raised and digestion completed when a clear liquid and white residue remained. After cooling the contents of the flask, 300 ml. of distilled water and then 125 ml. of 40 percent caustic soda were added. Taking all the necessary precautions, the ammonia formed was distilled and the distillate was collected into 50 ml. of four percent boric acid to which eight drops of mixed indicator (Brome cresol green and methyl red) were added. The distillate was back titrated against a standard deci-normal sulphuric acid. A blank digestion and distillation was run for the reagents used. From the amount of standard acid consumed for the back titration, the percent nitrogen in the sample was calculated by using the relationship

1 ml. 1 $\underline{N}$ H_2SO_4 = 0.014 g. nitrogen.

Wet ashing and chemical analysis

An aliquot portion of finely ground dry material or the residue left after moisture determination was used. The wet ashing was carried out in 125 ml. conical flask. Five ml. of nitric-perchloric acid mixture (1:1) for each 0.1 g. of the sample weighed was used for digestion. The flask was covered with a buret funnel and first heated on a hot plate kept in a hood and maintained at low temperature. When all the nitrogen peroxide fumes were given off, the temperature was raised and heating continued until the residue in the flask was white and about 1 to 2 ml. of the clear solution remained.

The flask was then taken off the hot plate, cooled to room temperature, and the contents transferred quantitatively to a 100 ml. (or 50 ml.) volumetric flask. From the washes of the repeated rinsing of the

flask, the volume of the extract was made to volume. After shaking well, the diluted acid extract was filtered through ashless dry filter paper (Whatman #42). The filtrate was collected and aliquot portions of this acid extract were used for calcium, magnesium, potassium, sodium, phosphorus, and iron determinations.

Calcium and magnesium.--These two were determined in one aliquot portion of the acid extract. In actual determination 10 ml. of acid extract was passed through cation exchange resin column (three inches of Dowex IX). The aliquot was washed through with 20 to 30 ml. of distilled water into a titration flask. One ml. of four percent potassium cyanide solution was added. This was followed by one ml. of four percent hydroxylamine hydrochloride and 8 to 10 drops of four percent potassium ferrocyanide and enough NH_4OH-NH_4Cl buffer to bring the pH to 10. After adding six drops of magnesium EDTA solution and six to eight drops of Eriochrome Black T indicator, the contents of the flask were titrated against 0.01 N EDTA solution to a blue color.

Another 10-ml. portion of the acid extract was again passed through the cation exchange resin column as above (the column was recharged with HCl and washed free of acid) and washed with distilled water. One ml. each of potassium cyanide, hydroxylamine hydrochloride, and potassium ferrocyanide were added. Enough 4 N sodium hydroxide was also added to raise the pH to 12 to 13. A pinch of Patton and Reeder indicator powder was added and titrated against 0.01 N EDTA solution as above. The first titration reading was for calcium plus magnesium while the second reading was only for calcium. The difference gave the reading for magnesium. Percent calcium and magnesium were calculated from the relationship

1 ml. of 1 N EDTA = 0.02 g. calcium

= 0.012 g. magnesium.

Potassium and sodium.--These two were determined by flame photometer (Perkin-Elmer Corporation, model #146). A single beam was used and butane-air mixture was employed for getting the excited characteristic flame of the metal under study.

The original test solutions were diluted fivefold before testing in the flame photometer. The instrument meter was calibrated against 40 ppm. potassium standard solution and one ppm. sodium standard solution. The instrument was operated on a regulated line of 110 volts.

Phosphorus.--The method followed is the one described by Jackson (1958). One ml. of the acid extract was taken into a 25 ml. volumetric flask. It was diluted with distilled water to about 15 ml. The pH of the solution was adjusted to about 3.0 by adding 2 N NaOH solution in the presence of two drops of 2,6 dinitrophenol indicator, till the color became yellow and again discharging the color by adding 2 N H_2SO_4. Two ml. of sulphomolybdic acid was added and finally color was developed with three drops of freshly prepared stannous chloride solution. The volume was made to the 25 ml. mark with water, and after mixing the optical density was measured against reagent blank, at 660 mμ in a Beckman model B spectrophotometer, which was operated on regulated voltage. From the standard curve prepared by using 5, 10, 20, and 40 gamas of P as pure KH_2PO_4, the concentration of phosphorus in the unknown was calculated.

Iron.--An aliquot portion of the acid extract (in most cases 15 ml.) was taken in a 25 ml. flask, and the pH was adjusted to 3.5 with 2 N sodium acetate solution; the amount was determined on a separate aliquot by using a glass electrode pH meter. One ml. of 10 percent

hydroxylamine hydrochloride solution was added and color was developed by the addition of 2 ml. of 0.2 percent orthophenanthroline reagent. Volume was made up to the mark with distilled water and mixed. After half an hour, optical density at 500 mu was read against the blank for reagents on a Beckman Spectrophotometer (model B) using one cm. cuvettes. The instrument was operated on a regulated line voltage and was allowed to warm up for 30 minutes before taking readings.

A solution of hydroxylamine hydrochloride keeps well when stored in a refrigerator. Fresh solutions were therefore prepared only after a month or earlier if exhausted. Orthophenathroline solution was also stored in the refrigerator, but not for more than a week.

The amount of iron in the test solution was calculated from the standard curve prepared from standards containing 2, 5, 8, 10, 12, and 15 gamas of pure iron (electrolytic iron having purity of 99.8 percent, obtained from Baker Chemical Company, Philipsburg, N. J.).

pH.--This was determined using a line operated glass electrode pH meter (Beckman Ac model No. H2). The instrument, after warming up, was standardized against a buffer solution of pH 7.0 and then samples of expressed leaf sap were tested. The temperature compensation knob was adjusted to the temperature of the test liquid. The instrument read directly the pH values.

Details of the preparation of samples of leaf juice are given under respective experiments in the next chapter.

Oxidation-reduction Potential

A platinum electrode (Beckman #281) and a calomel electrode (Beckman #4970) were used as two half cells. The test sample was placed in a small flask stoppered by a snugly fitting rubber cork with four holes. Two electrodes passed through the cork and dipped into the sample. The

other two holes were used for the inlet and outlet of nitrogen gas. The setup of the whole assembly is shown diagramatically in figure 1. Nitrogen gas from the cylinder was passed through saturated pyrogallol solution to remove oxygen, if any, in the incoming nitrogen gas.

A slow stream of nitrogen gas was let in and bubbled gently through the test sample for five minutes. The electrodes were connected to a potentiometer (the Beckman pH meter with the switch in "MV" position was used as a potentiometer) and EMF of the cell was read on the scale of the instrument in millivolts. Simultaneously, the sample was also tested for its pH. From the observed EMF and pH the oxidation-reduction potential of the sample was calculated using the equation

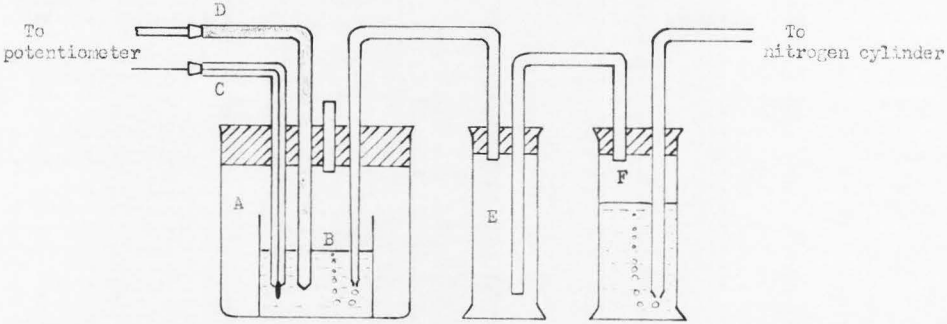
$$Eh_{(pH\ 7.0)} = (\text{EMF observed} - \text{EMF of calomel electrode}) + 0.06\ (7 - \text{sample pH}).$$

EMF of the calomel electrode was taken as 0.246 V. The results are expressed as Eh at pH 7.0.

Enzyme Activity

Catalase

The activity of the catalase enzyme preparation was assayed from an aliquot portion of fresh leaf sample. One gram of the sample was weighed on a torsion balance and transferred to a clean chilled porcelain mortar containing five ml. tris buffer (0.2 M tris in 0.3 M sucrose solution, pH adjusted to 7.0). A pinch of acid-washed fine sand was added and the leaf material triturated until a fine suspension of leaf homogenate was obtained. After allowing the sand to settle, the suspension was tilted on one side and transferred to a 50 ml. flask by means of a mechanical pipette. The mortar was rinsed several times with buffer solution and all washings transferred to the flask and finally made to volume.



A Outer vessel
B Inner vessel containing the test liquid (leaf juice)
C Platinum electrode
D Calomel electrode
E Catch
F Vessel containing saturated solution of pyrogallol

Figure 1. Assembly for oxidation-reduction potential measurement

Catalase activity was determined manometrically using a Warburg apparatus provided with a temperature control and shaking mechanism. Catalase in the leaf homogenate was allowed to react with hydrogen peroxide at a constant temperature and volume and the liberated oxygen was measured in the manometer.

One ml. of the leaf extract and 0.2 ml. of 0.1 M H_2O_2 were placed in the main vessel and side arm, respectively, of the Warburg flask. After equilibrating for five minutes, H_2O_2 from the side arm was tipped into the vessel containing the enzyme preparation. This was the zero time. Manometer readings were recorded at an interval of two minutes each for a period of 20 minutes. The thermobarometer contained one ml. of buffer solution and 0.2 ml. of 0.01 M H_2O_2. The reaction was allowed to proceed at a constant temperature of 30° C and a shaking rate of 120 cy/min.

At no stage of the preparation of enzyme material was there contamination from metal. All the operations, including weighing, were carried out in the cold room and only glass-redistilled water was employed. Results are expressed as microliters of oxygen liberated from 1 mg. dry weight within 20 minutes.

Peroxidase

One gram leaf sample was weighed on a torsion balance and triturated in a chilled mortar along with a pinch of acid-washed sand and five ml. of redistilled water. The homogenate was made to 50 ml. and after mixing well was strained through four folds of cheese cloth. Peroxidase activity was determined by measuring the change in optical density of pyrogallol solution in the presence of enzyme preparation and H_2O_2 at pH 4.5.

Twenty ml. of acetate buffer, pH 4.5, was taken into a 23 ml.

colorimeter tube. One ml. of the enzyme preparation and 0.5 ml. of 0.05 M H_2O_2 were added. At zero time 0.5 ml. of one percent aqueous solution of pyrogallol was added, and the change in optical density after 10 minutes was measured at 425 mμ using the blue filter with Fisher electrophotometer (AC model). The instrument was operated on a regulated line voltage of 110 volts to avoid any fluctuation in voltage affecting the instrument reading. The reaction was carried out at room temperature. The results are expressed in arbitrary units of the instrument dial.

Cytochrome oxidase

One gram of the leaf sample was weighed on a torsion balance and triturated in a chilled mortar in the presence of five ml. tris buffer (0.05 M tris in 0.3 M sucrose solution, pH 7.0) and with a pinch of acid-washed sand. The leaf homogenate was made to 50 ml. volume and centrifuged for 20 minutes at 1000 x g. in a centrifuge kept in the cold room but with remote control from outside (Ivan Sorval Type SS-3). The supernatant was used for enzyme activity measurement.

The activity of the enzyme preparation was measured manometrically using the Warburg apparatus. In the side arm 0.1 ml. of 3 percent hydroquinone was placed. The main flask contained 0.1 ml. of cytochrome c solution (10 mg./ml.) and one ml. of the enzyme preparation. In the center well was put 0.2 ml. of 20 percent KOH. After equilibrating to 30° C for 10 minutes, at zero time, hydroquinone from the side arm was tipped. Manometer readings, thereafter, were recorded at an interval of 30 minutes for a total period of 150 minutes. The thermobarometer contained 0.1 ml. of buffer solution instead of cytochrome c in the flask. The results are expressed as microliters of oxygen consumed per hour per 1 mg. dry weight.

Dehydrogenase activity

This was measured by observing the rate of reduction of 2,3,5-triphenyltetrazolium chloride. A set of five discs of 1 cm. diameter each was punched from leaf samples and transferred to small vials equipped with screw caps. The vials contained 2 ml. of 0.1 percent aqueous solution of tetrazolium salt. At the end of five days the insoluble carmine red triphenyl formazan formed was dissolved in 2 ml. methyl alcohol and extracted with 10 ml. toluene by shaking vigorously. After centrifuging, the supernatant colored toluene layer was taken with a mechanical pipette and the absorbance measured at 490 mμ against a toluene blank extract using the Beckman spectrophotometer. The results are expressed in arbitrary units of the instrument meter.

Protein

This measurement was made by adopting the Folin-phenol reagent method (Lowry et al., 1951). In all cases, the leaf homogenate or the protein fractions were diluted in such a manner that one ml. of the sample was used for color development, with the reagents making the final volume to 6.5 ml. and the optical density not higher than 0.5 when read at 650 mμ on a Beckman model B spectrophotometer.

One ml. of the diluted homogenate was taken into a test tube to which 5 ml. of protein reagent was added. The mixture was maintained at 37° C for 30 minutes in a constant temperature water bath. Later, 0.5 ml. of phenol reagent was added and the mixture shaken vigorously immediately. Again the mixture was maintained at 37° C for 20 minutes in the water bath. The optical density of the blue color developed was measured at the end of 20 minutes, as early as possible, against a reagent blank at 650 mμ in the Beckman Spectrophotometer. The

concentration of protein was then calculated by referring to the standard curve prepared using standard casein solutions as below.

On a chemical balance, 0.05 gm. of pure casein powder was accurately weighed in a tared watch glass and then transferred to a clean mortar. The casein powder was ground with water to form a thin paste and dissolved completely by adding 0.5 ml. of 1 N NaOH. The casein solution was then transferred quantitatively to a 100 ml. flask by rinsing the mortar and pestle several times with a jet of water and pouring the washings to the flask. The volume was made up to the 100 ml. mark. After mixing well, 2, 5, 10, 15, and 20 ml. of the casein solution were pipetted into 50 ml. flasks. When diluted to the mark, each ml. of the solution respectively contained 20, 50, 100, 150, and 200 gamas of protein as casein. Color was developed with one ml. of these solutions as above and the optical density was measured at 650 mμ. A graph was drawn by plotting optical density against concentration.

Radioactivity counts

A five ml. aliquot portion of the protein fractions obtained by differential centrifugation was taken into a test tube and dried in an oven. The radioactivity was then measured by taking counts using a scintillation counter (model Ds-1 of Nuclear Instrument and Chemical Corporation) connected to a scaler (model 183 of the Nuclear Instrument and Chemical Corporation). The scaler was run for four to five hours before taking counts of the samples. A high voltage of 750 volts was used in the plate circuit, and in most cases background counts were taken before and after the sample counts. An average of the two background counts was used for computing the net sample counts. Details of the treatment of plants with Fe59 are described elsewhere.

The method of raising plants in solution culture and the method for differential centrifugation are described in the methods for the various experiments.

CORN EXPERIMENTS

Experiment 1

This experiment was made to study the nutrition of normal and chlorotic plants. In the literature reviewed it was noticed that the iron content of chlorotic leaves was the same or even higher than in the normal ones. This apparent anomoly was attributed either to the surface contamination or to the stunted growth of plants in which the absorbed nutrients are concentrated in the leaf. If two sets of plants could be grown under favorable conditions of growth medium, temperature, and light--one set with a normal complement of iron and the other with no iron--the leaf analysis of such plants might reveal the true picture of nutrient status of chlorotic plants. An attempt was therefore made in this direction.

Methods

Corn plants were grown in solution culture. Uniform healthy seeds were selected and, after disinfecting with one percent chlorox solution, were allowed to germinate in a germinator. Seven days later, healthy seedlings were selected for uniformity and transplanted into mason gallon jars of nutrient solution. The jars were painted outside with black paint followed by aluminum paint. Three seedlings were planted in each jar. The seedlings were held in position by split corks padded with cotton wool, fixed in waxed paper lids fitted on the tops of the culture jars.

As mentioned earlier, deionized distilled water was used for preparing culture solutions. The formula used is essentially the same as

Hoagland's No. 2 (Hoagland and Arnon, 1938) except for the molarity of the stock solutions. The two solutions are compared in table 1.

Table 1. Hoagland's No. 2 solution and modified formula used in the present study

Hoagland's No. 2 solution[a]		Modified solution	
Stock solutions	ml. in a liter of nutrient solution	Stock solutions	ml. in a liter of nutrient solution
1 M $NH_4H_2PO_4$	1	0.2 M $NH_4H_2PO_4$	5
1 M KNO_3	6	1.2 M KNO_3	5
1 M $Ca(NO_3)_2$	4	0.8 M $Ca(NO_3)_2$	5
1 M $MgSO_4$	2	0.4 M $MgSO_4$	5

[a]Hoagland and Arnon, 1938.

The modified solution has the same final concentration of different nutrients as Hoagland's No. 2. However, it has the advantage of having one measure for all stock solutions which makes it easy to dispense. Secondly, it helps in quick preparation of $NH_4H_2PO_4$ and $MgSO_4$ stock solutions, whose M quantities normally take time to dissolve completely.

Minor elements were supplied as in Hoagland's recommendation (Hoagland and Arnon, 1938) and 5 ppm. of iron was given in the form of Sequestrene 330-Fe iron chelate, containing 10 percent iron (Geigy Agricultural Chemicals). The pH of the culture solutions was adjusted to 5.5.

The culture solutions were aerated continuously, at a fairly uniform rate, by means of glass tubes with capillary ends connected to the compressed air line. The solutions were changed every four days.

The plants were grown in a growth chamber of controlled temperature and lighting conditions. A 16-hour light period was given to the plants.

In all, there were 20 jars with three plants in each. Seven days after transplanting, one set of 10 jars received a normal dose of iron in culture solution while a second set of 10 jars got no iron. These plants consequently became chlorotic, and except for the chlorosis, they were all similar to the green ones in respect of height, growth, and condition of roots. In table 2 is given the number of completed leaves of plants in each jar of the two sets of plants.

Three weeks after transplanting, plants were sampled for analytical work. There were two sets of plants, one green and the other chlorotic. Each set of 10 jars, having three plants in each jar, was divided into five replications of two jars each. Thus, each replication had 2 x 3 = 6 plants. For sampling, two jars, that is one replication, were taken at a time. Sampling was done in a cold room to avoid loss of moisture during the sampling process. The second, third, and fourth leaf from the top of corn plants were harvested. Any surface dust on the leaves was wiped off first with a dry and then with a moist clean foam-rubber sponge. Two-inch portions of tips and bases were removed along with the entire mid-rib. The remaining portion of the leaf was made into small pieces approximately one-half inch in length. After mixing well, aliquot portions were weighed for various determinations. Moisture, iron, phosphorus, calcium, magnesium, sodium, and potassium were determined in one aliquot sample of 10 grams weighed on a chemical balance. Nitrogen and protein were analyzed separately from a one-gram sample for each, while a 20-gram sample was weighed on a torsion balance for expressing leaf juice for pH and oxidation-reduction potential.

Table 2. Fully developed leaves per jar containing three healthy or chlorotic corn plants grown in nutrient solution with or without added iron

Healthy		Chlorotic	
Replication	Number of leaves	Replication	Number of leaves
1	24	11	20
2	23	12	24
3	21	13	23
4	24	14	24
5	23	15	24
6	22	16	22
7	23	17	22
8	23	18	23
9	23	19	22
10	24	20	24
Mean	23.0	Mean	22.8

The methods used have already been described for the individual analyses. For expressing sap or juice from leaves, the sample was stored for 24 hours in dry ice. After allowing the sample to thaw at room temperature, it was pressed in a gun-metal cylinder and plunger coated with petroleum jelly. A hydraulic press (Carver Laboratory press) was used with a pressure of 14000 lb./sq. inch held for two minutes. The juice was collected in a plastic container and immediately transferred to a centrifuge tube. Nitrogen gas was bubbled through the juice and after stoppering it was stored in a refrigerator until used for pH and oxidation-reduction potential measurements. Methods for these determinations have already been described elsewhere.

Results and discussion

The results of analysis are shown in table 3. They are the mean values of five replicate samples.

Chlorotic leaves seem to contain more moisture to an extent of about 18 percent. This accounts for the more succulent nature of such leaves which could be felt while grinding the leaf tissue in a mortar or while homogenizing the sample for fractionation. Due to the deficiency of chlorophyll, the carbohydrate metabolism is reduced and cell wall material is not deposited to the same extent as in the case of normal leaves.

The moisture levels are not in line with those of Pound and Welkie (1958) who noticed increased dry and fresh weight, leading to low moisture content, in the case of iron deficient tobacco leaves. Their results were on the unit area basis. However, the tobacco leaves in iron deficient and virus effected plants were smaller than the normal ones. In the present experiment, the chlorotic leaves were of the same

Table 3. Moisture, nitrogen, protein, mineral composition, Eh, and pH of healthy and chlorotic leaves of corn plants grown in nutrient solution with or without added iron[a]

Replication	Moisture	Total N	Protein	Protein/N	Ca	Mg	K	Na	P	Fe	K/Ca	P/Fe	Eh (pH 7)	pH
	percent	me./100 g.	percent		me./100 g.	me./100 g.	me./100 g.	me./100 g.	me./100 g.	me./100 g.			volts	
Healthy														
1	675.3	304.6	29.46	6.91	18.0	20.4	100.0	1.0	4.94	0.23	6.06	21.48	0.555	5.63
2	716.7	320.7	34.15	7.64	22.0	19.4	100.0	0.6	4.52	0.22	4.54	20.54	0.553	5.60
3	706.3	315.8	28.96	6.55	15.0	15.9	100.0	0.8	4.19	0.20	6.67	20.95	0.556	5.70
4	674.5	303.0	32.45	7.65	14.0	17.7	95.0	0.6	5.16	0.25	5.28	20.64	0.551	5.61
5	662.0	300.2	32.40	7.56	24.0	17.1	99.0	0.6	4.32	0.23	3.96	18.78	0.556	5.70
Mean	687.6	310.1	31.48	7.26	19.5	18.1	99.6	0.7	4.63	0.23	5.29	21.84	0.554	—
Chlorotic														
1	784.8	281.7	21.10	5.35	11.0	11.9	95.0	0.7	3.39	0.02	8.64	169.5	0.558	5.70
2	833.8	293.3	22.78	5.55	13.0	9.6	103.0	0.7	3.39	0.02	7.92	169.5	0.556	5.70
3	821.1	300.1	25.17	5.99	8.0	13.5	105.0	1.1	3.32	0.02	13.13	166.0	0.554	5.70
4	806.1	291.6	23.67	5.86	7.0	13.1	92.0	0.9	2.58	0.02	13.14	129.0	0.554	5.70
5	806.7	290.4	25.14	6.19	10.0	13.6	105.0	0.6	3.55	0.02	10.5	177.5	0.557	5.70
Mean	810.6	291.4	23.57	5.77	9.8	12.3	100.0	0.8	3.25	0.02	10.67	162.3	0.556	—

[a]Expressed on a dry weight basis.

size and shape as the normal leaves. An epinastic effect is commonly observed in tobacco leaves affected by physiological and virological diseases. This might be one of the reasons of increased green and dry weights of chlorotic leaves noticed.

There was no appreciable difference in the nitrogen content of healthy and chlorotic leaves. The Kjeldahl method includes both protein and non-protein nitrogen. A slightly lower nitrogen content observed in the chlorotic leaves may be explained as due to the reduction in the synthesis of chloroplastic protein in the absence of an iron supply.

In contrast with nitrogen, the protein level for chlorotic leaves was far below that of the normal leaf. The reduction was almost 25 percent. In spinach leaves nitrogen in the form of chloroplastic protein makes up roughly 40 percent of the total nitrogen (Bonner, 1950). Since the chlorotic leaves were deficient in chloroplasts, the protein level became low. It appears from the work of Jacobson (1945) and Bennett (1945) that iron which is active in chlorophyll formation is that bound in organic combination actually in the chloroplasts and is essential for initiating synthesis of chloroplastic proteins in leaves. Iron deficiency has therefore suppressed the protein synthesis in chlorotic leaves. However, due to the sufficient supply of nitrogen in the culture solution, its absorption continued in spite of a low demand in leaves for protein synthesis. This resulted in the accumulation of non-protein nitrogen in chlorotic leaves, as indicated by a low protein to nitrogen ratio. Similar observations were made by several workers including Bennett (1945), Iljin (1951), and DeKock and Morrison (1958).

DeKock and Morrison (1958) correlated free amino acids with the P/Fe ratio, the former being high when P/Fe ratio was high and declining as the ratio declined. This, they said, was true irrespective of the

cause of chlorosis. In the present experiment also, the P/Fe ratio was higher in chlorotic leaves than in normal ones. This might have contributed to the presence of non-protein nitrogen in higher concentration in chlorotic leaves.

Considering calcium and magnesium in the two types of leaves, an appreciable reduction in both of these divalent cations was observed in chlorotic leaves. Calcium was reduced by 50 percent and magnesium by 33 percent. Much of the calcium in leaf tissue is used in cell wall material. As a result of reduction in photosynthesis due to lack of chlorophyll in chlorotic leaves, the carbohydrate synthesis lagged. Since there was not much demand for calcium in building cell wall material, probably its uptake was low. The results for calcium do not corroborate the results of other investigators. Both Iljin (1952) and DeKock and Morrison (1958) have reported a higher accumulation of calcium in chlorotic plants. However, Lindner and Harley (1944) did not notice increases in calcium in chlorotic leaves. Olson (1950) and Wadleigh and Brown (1952) also reported that there was no difference in the calcium content of chlorotic and non chlorotic leaves. Thus it is difficult to explain the discrepancy between the results of these authors. Probably pH, moisture conditions, and concentration of other elements with respect to calcium are involved in the nutrition of calcium.

Magnesium is an important constituent of the chlorophyll molecule. A large proportion of the magnesium present in green leaves is, therefore, in chlorophyll. Since synthesis of chlorophyll itself is low in chlorotic leaves, magnesium was not absorbed to the same extent as in the case of normal plants. In the literature both increases and decreases in magnesium content of chlorotic leaves are reported. Olson

(1950) found that green leaves of sorghum had a lesser magnesium content than chlorotic ones. In the data published by DeKock (1958) on the amino acid content of several chlorotic and non chlorotic plants, both increases and decreases in magnesium contents are seen. However, the differences are not appreciable. In alkaline soil, sometimes, magnesium concentration is so high that the element is absorbed in quantities sufficient to cause toxicity. This might be one of the possible reasons for a higher concentration of magnesium in chlorotic leaves, as reported by Olson (1950).

Contrary to the findings of many investigators, the uptake of potassium and sodium were not materially affected as a result of iron deficiency. This may be explained on the basis of the method of expressing the results. If the composition is expressed on the percent basis, naturally, the smaller the size of the plant, the higher will be the figure for percentage composition for any element. In the normal growth of plants, the absorbed nutrients are proportionately distributed throughout the entire plant and, due to more dry weight, percentage composition decreases. However, if the total uptake of the two plants are compared, the chlorotic should invariably show less. Most of the mineral ions may have been absorbed before the chlorosis developed in this experiment.

As one could expect, the iron level in chlorotic leaves was reduced to a great extent (91 percent). Compared with normal leaves, phosphorus was also reduced in chlorotic leaves, but only by 30 percent. One week after transplanting the iron supply was completely withheld in the case of chlorotic plants. However, phosphorus composition of the nutrient solution was not altered. Once again, the reduced uptake of phosphorus by chlorotic plants may be attributed to the reduction in carbohydrate

metabolism as a result of low photosynthetic activity. In addition to its presence in phospholipids and nucleic acids, the role of phosphorus is quite significant in phosphorylation and in forming energy rich phosphate bonds in carbohydrate metabolism. Since the latter process is slow in chlorotic leaves, the demand for phosphorus in the photosynthetic organ was less which consequently reduced its absorption.

The results for iron and phosphorus are not in agreement with those of several investigators. Iljin (1952) detected, in certain cases, higher iron concentration in chlorotic leaves than in nonchlorotic ones. Both increases and decreases in iron, as well as phosphorus, were reported by DeKock and Morrison (1958) in their analysis of different plant species affected by chlorosis. The chlorosis developed in plants studied by these authors probably resulted from different causes. As such, the conclusion drawn by one author may not be applicable in other cases. The situation under lime-induced chlorosis would be different from the one developed by simple iron deficiency. In the case of lime-induced chlorosis, the absorbed iron might be inactivated in the leaf, thus showing high results for this element. Iljin (1952) states that lack of iron does not appear to be the main cause of lime-induced chlorosis.

It was thought that there would be a profound change in the oxidation-reduction potential as a result of iron deficiency. However, this does not seem to be the situation. Perhaps there are systems other than the simple Fe^{++}------Fe^{+++} system which contribute substantially to the oxidation-reduction potential of the leaf sap. There does not appear to be much available information in the literature on this subject.

No significant difference in the pH of leaf sap was noticed. The

cell sap is generally highly buffered by the presence of organic compounds, and consequently changes in mineral composition of leaves may have very little or no influence at all on the reaction of the leaf sap. The figures for pH values agree with the ones reported by Hurd-Karrer (1939) as a range from pH 5.5 to 6.5 for the majority of plants. Oserkowsky (1933) noticed no significant difference between H^+- ion concentration and the iron level in tracheal sap of green and chlorotic branches from pear trees in the same orchard. However, Rogers and Shive (1932) established a direct correlation between the pH of the tissue fluid of plants and the amount of total and soluble iron present in the tissue. In the case of lime-induced chlorosis, excess accumulation of organic acids in leaves was observed (Iljin, 1952; DeKock, 1955). But it is not known whether these acids were present in free form or as salts of cations. Placing these facts side by side, it would be difficult to understand how iron is inactivated even in the presence of accumulated acids in chlorotic leaves. The presently available data are insufficient to explain the situation.

Molecular ratios of cations are found to be good indices for characterizing physiological disturbances in plants. At least two mineral element ratios have been found upset in chlorotic leaves. DeKock and Hall (1955) suggested one more ratio of magnesium-potassium in addition to the commonly referred potassium-calcium and phosphorus-iron ratios. Considering the results in table 3, the chlorotic leaves exhibited a higher ratio both for potassium-calcium as well as for phosphorus-iron. Reported results of Lindner and Harley (1944), Wadleigh and Brown (1952), DeKock (1955), and DeKock and Morrison (1950) show high ratios for these elements in chlorotic leaves. In the results of these authors, there was an excess accumulation of potassium and

phosphorus respectively over calcium and iron in chlorotic leaves. In the present experiment, no such accumulation could be seen, but the increase in the two ratios is attributed to low absorption of calcium in one case and the deficiency of iron in the other. It is doubtful how far these ratios could be used in explaining the causes of chlorosis. Thorne et al. (1950) also suggested that the disturbed potassium-calcium ratios are a result of chlorosis rather than a cause of it.

The implication of a reduced protein-nitrogen ratio has already been discussed. It has been said that nitrogen in chlorotic leaves is not completely utilized in protein synthesis and, therefore, may accumulate in the form of non-protein nitrogen which accounts for this decreased ratio observed in chlorotic leaves.

Experiment 2

This experiment was planned to study the effect of iron chlorosis on enzyme activity and the status of iron and protein in various leaf fractions.

Methods

Culture and sampling procedures.--Corn plants were raised in solution culture by the technique followed in the previous experiment. Three weeks after transplanting, leaf samples were taken for measuring enzyme activity and for fractionation of leaf proteins. Methods employed in the sampling for these differed from those of the previous experiment and will be explained.

There were two treatments in this experiment also, one with iron and the other without iron. Each treatment had five jars with two plants in each. This constituted one replication. In all, six replications were run. The total number of plants for sampling each treatment per replication was thus 5 x 2 = 10 plants. Before sampling, the

plants were removed to the cold room. Three leaves, the second, third, and fourth from the top of each of the 10 plants, were harvested and pooled. The surface dust, if any, was wiped off by means of a clean sponge. Careful consideration was given for proper sampling of leaf tissue since the enzyme activity, especially that of catalase, has been found to vary markedly from the base to the tip of the leaf (Euler, 1948). Four-inch portions from the tip and base of the leaves were removed along with the midrib, and the remaining part was made into small pieces of approximately one-half inch in length. All this operation was done by clean hands and at no stage was there any contamination from metal.

Out of the composite sample, one set of aliquots was weighed on a torsion balance. From the remaining bulk of the sample, 25 g. was weighed on a torsion balance for protein fractionation. Catalase activity was measured immediately after sampling, while the rest of the samples were properly labelled and stored in a deep freeze at -20° C. Peroxidase and cytochrome oxidase activities were measured the day following the harvest. The protein fractionation was done in due course of time.

In two of the replications, corn plants were treated with Fe^{59} for tracing it in different fractions of leaf proteins. A 0.5 percent solution of $FeSO_4 \cdot 7H_2O$ was prepared. To about 100 ml. of this solution 1500 microcurries (calculated from the activity at the time of shipment of the radioactive compound) Fe59 as $FeSO_4$ was added. One drop of concentrated H_2SO_4 was also added and the final volume made to 150 ml. After mixing well, 5 ml. of this was applied to each gallon jar containing fresh culture solution and to which no iron compound was added previously. This supplied about 50 microcurries Fe59 to each gallon of

culture solution. The corn plants were allowed to absorb Fe^{59} for a period of 48 hours, after which they were sampled for protein fractionation and the radioactivity measured by the method already described.

From another set of corn plants raised separately, the third leaf from the top of the plant was sampled. After removing the surface dust and trimming the tips and bases as above, discs one cm. in diameter were punched for measuring fresh and dry weight per unit area, respiration, (results for these two not reported here) and dehydrogenase activity. In this case there were only five replications with two plants each.

Fractionation of leaf proteins.--The sample that was stored in the deep freeze was divided into half by weighing on a torsion balance. The leaf tissue was allowed to thaw at room temperature for 15 minutes and then taken to the cold room. The sample was transferred to a chilled stainless steel jar (200 ml. capacity) lined with teflon. The leaf tissue was allowed to soak in 50 ml. distilled water for five minutes. It was then homogenized for 1½ minutes in an omnimixer (Servall model OM) operated at full speed. The jar was kept in a crushed ice bath to prevent any rise in temperature during homogenization. The slurry was filtered through four folds of cheese cloth. The residue on the cloth was squeezed to obtain as much juice as possible. Fresh and dry weights of the residue were recorded. The other half of the leaf tissue was treated likewise. The homogenate from the two were mixed and subjected to fractionation by differential centrifugation. Relative centrifugal forces up to 17000 x g were obtained using a Servall type SS-3 super-speed centrifuge with remote speed control. The centrifugation was done in the cold room. Nylon 50 ml. tubes were used in the rotor head. For higher centrifugal forces, the refrigerated Spinco model L ultra-centrifuge with rotor head #40 was used. In this case 10 ml. celluloid

tubes were employed.

After spinning the sample at appropriate speed, the supernatant was transferred to another tube by means of a mechanical pipette for successive centrifugation. Each sediment was washed once with water and recentrifuged, transferring the wash to the succeeding fraction. The pellets obtained in different fractions were resuspended in water by homogenizing in a Ten Broeck homogenizer, making the final volume to 50 ml. The clear supernatant obtained in the last fraction was, however, made to 150 ml.

Aliquot quantities of the resuspended fractions were used for protein and iron determinations. A scheme of fractionation procedure employed is summarized in figure 2.

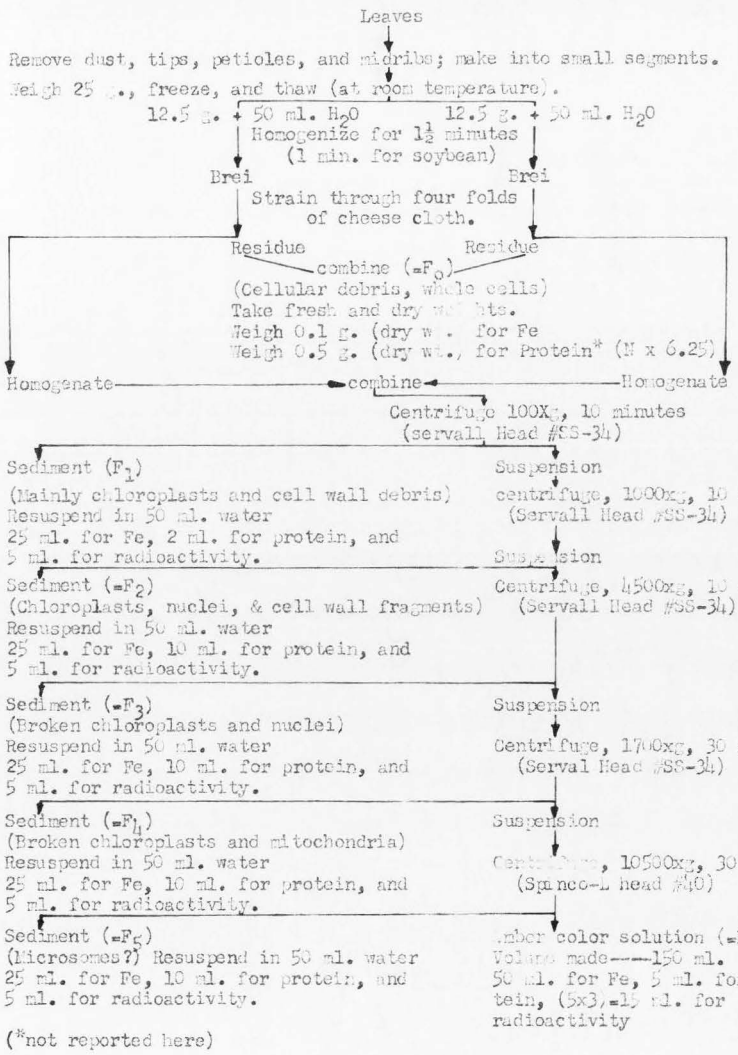
Results and discussion

Effect of iron chlorosis on enzyme activity.—Catalase is a conjugated protein containing an iron porphyrin as its prosthetic group. A reduction in the iron supply should naturally be impressed upon the catalase activity. As can be seen from table 4, iron chlorosis in corn leaves had an appreciable effect on catalase activity. In comparison

Table 4. Catalase activity[a] in corn leaves of plants grown in nutrient solutions with or without added iron

Replications	Healthy	Chlorotic
	µl. oxygen/mg. dry wt./20 min.	
1	8.05	0.79
2	8.84	1.67
3	9.57	1.23
4	8.93	1.86
5	8.44	2.00
6	9.45	1.49
Mean	8.83	1.51

[a]Oxygen liberated in presence of enzyme preparation from hydrogen peroxide.



(*not reported here)

Figure 2. Flow sheet for fractionation of leaf proteins

with the normal leaves, the enzyme activity in the chlorotic ones was depressed by about 83 percent. Bailey and McHargue (1944) noticed considerable uniformity in the enzyme responses to the nutrition of minor elements. Eyster (1950) compared the catalase activity of corn seedlings varying in chlorophyll pigment: albino, yellow, and green seedlings. His observations were that the albino seedlings had the least activity while the green ones had the maximum and yellow ones had intermediate. A series of hybrid strains of corn seedlings, when arranged from darkest green to lightest green, showed progressively less catalase activity in this order. Brown (1953) also found that catalase activity was lower in chlorotic plants than in normal green ones. McClendon (1953) noticed maximum catalase activity in chloroplast fraction of tobacco leaves. All these findings substantiate the results of the present experiment, as far as the catalase activity is concerned.

Appleman (1952), on the other hand, found higher catalase activity in etiolated barley seedlings than in green ones. However, it may be noted that in the experiment of Appleman, iron was not a limiting factor to the seedlings. Catalase and iron porphyrin synthesis in the etiolated barley seedlings was neither interferred with nor reduced. On the other hand, the author noticed that when the etiolated seedlings were exposed to light, greening started in the leaves but catalase activity decreased. This demonstrated that synthesis of an iron enzyme porphyrin, catalase, and the synthesis of chlorophyll were two independent processes, but for both these processes iron supply was essential. In the present experiment the corn plants developed chlorosis as a result of the deficiency of iron. This lack of iron limited the synthesis of chlorophyll as well as the catalase enzyme. These results therefore cannot be compared with those of Appleman (1952).

Results of the effect of iron chlorosis on peroxidase and dehydrogenase activities are shown respectively in tables 5 and 6. Since comparitive values rather than absolute amounts were considered satisfactory for the purposes of this investigation, the data are presented in arbitrary units of the instrument.

Table 5. Peroxidase activity[a] in corn leaves of plants grown in nutrient solutions with or without added iron

Replications	Healthy	Chlorotic
	percent absorbance[b]	
1	18.1	7.7
2	17.1	8.0
3	18.6	7.0
4	18.2	7.4
5	17.8	10.2
6	17.3	8.2
Mean	17.9	8.1

[a]Change in optical density of pyrogallol solution by enzyme preparation in presence of hydrogen peroxide.

[b]In units of instrument dial.

Table 6. Dehydrogenase activity[a] in corn leaves of plants grown in nutrient solutions with or without added iron

Replications	Healthy	Chlorotic
1	0.800	0.075
2	0.7600	0.254
3	0.9400	0.416
4	0.8600	0.280
5	1.400	0.38
Mean	0.9520	0.281

[a]Absorbance at 490 m μ, of reduced tetrazolium salt extract in toluene.

There is a phenomenal reduction in the peroxidase activity as a result of iron chlorosis. The change in percent absorption of

pyrogallol solution, due to peroxidase activity, is from 17.9 percent to 8.1 percent. This is almost 55 percent reduction when compared with the normal. Peroxidase is also an iron enzyme and the deficiency of iron in the chlorotic leaves might have considerably reduced its synthesis in the leaf tissue. These results are in complete harmony with those of Brown (1953) and Brown and Hendricks (1952). However, in one of his experiments Brown (1953) did not notice a significant change in the peroxidase activity as a result of lime-induced chlorosis. In the former case, the plants which the author studied included corn while in the latter experiment his test plants did not include corn. Bailey and McHargue (1944) noticed that in the presence of more than 1 ppm. manganese in the nutrient solution, the peroxidase activity in alfalfa was depressed. Since iron and manganese are closely related physiologically in the plant, peroxidase being an iron-porphyrin, it may be that manganese in some unknown fashion affected the iron in the peroxidase molecule.

The three enzymes catalase, peroxidase, and oxidase, all catalytically decompose H_2O_2 and liberate oxygen. These enzymes are normally present in green leaves. Since in the present investigation both catalase and peroxidase activities were measured in the presence of H_2O_2, the question of interference from one another naturally is posed. Apart from the fact that the chemistry of the catalytic reaction of these enzymes is different, the optimum conditions for their activities also differ. Advantage of this property was taken in the measurement of peroxidase activity in the acetate buffer of pH 4.5. At this pH peroxidase activity is not prevented while interference from catalase and oxidase is eliminated.

The 2, 3, 5-triphenyltetrazolium chloride has been used extensively

as an indicator of high metabolic activity in a variety of plant tissues. There are several enzymes which can be said to be indicative of metabolic activity in the tissue. However, the property that oxidases transfer hydrogen directly to oxygen while dehydrogenases transfer hydrogen to an acceptor has made possible the use of the tetrazolium salt in the test for dehydrogenase activity.

Table 6 shows the effect of iron chlorosis on the dehydrogenase activity of the leaf tissue. Since the amount of extractable reduced tetrazolium salt was more (as indicated by the higher absorbance reading of about 55 percent) in healthy leaves than in the chlorotic ones, it may be concluded that the iron deficiency also affected markedly the dehydrogenase activity. Hewitt and Agariwala (1952) used the tetrazolium salt reduction test as an indicative of molybdenum deficiency in plant tissues. They observed that plant tissues grown with low molybdenum showed more reduction of the salt than normal plant tissues. Brown (1954), however, found more reduction of tetrazolium salt in the nodes and leaf veins of lime-induced chlorotic plants of corn. In the lime-induced chlorosis, absorbed iron in the tissues is inactivated or precipitated by change of $F^{++} \rightleftharpoons F^{+++}$, the reaction which involves the transfer of electrons. Since tetrazolium salt is a good acceptor of electrons, the reduction of this salt observed by Brown (1954) in the case of lime-induced chlorosis may, therefore, be attributed to the precipitation of ferric iron compounds rather than to the higher hydrogenase activity.

Cytochrome oxidase is one of the respiratory enzymes which catalyzes the oxidation of cytochrome c which has previously been reduced. Like other cytochromes, this oxidase is also a heme protein,

that is, an iron-porphyrin compound. The assay of this enzyme has been used as a measure of respiratory activity and the rate of metabolism in the tissues. In the present experiment it was noticed that iron chlorosis caused a reduction in the cytochrome oxidase activity (table 7). Compared with the above described enzymatic activities, the cytochrome oxidase activity seemed to have not been affected to a great extent. McClendon (1953) reported that higher activity of cytochrome oxidase was associated with smaller particles sedimenting at a higher centrifugal force, and lesser activity with the chloroplast fractions of the leaf tissue. The smaller particles were identified as mitochondria. Weinstein and Robbins (1955) found that low levels of iron retarded the cytochrome oxidase activity in sunflower plants. This suggests that although cytochrome oxidase is not a constituent of chloroplasts, iron supply is necessary for its normal functioning. Since there is more than one terminal oxidase involved in the transfer of electrons in the respiratory system, the effect of iron deficiency on cytochrome oxidase does not seem to be very great.

Table 7. Cytochrome oxidase activity[a] in corn leaves of plants grown in nutrient solutions with or without added iron

Replications	Healthy	Chlorotic
	ul. O_2/mg. dry wt./hr.	
1	1.43	1.19
2	1.71	1.18
3	1.58	1.28
4	1.32	1.13
5	1.53	1.06
6	1.60	1.13
Mean	1.53	1.15

[a]Net oxygen consumed in presence of enzyme preparation, hydroquinone, and cytochrome c.

Protein in fractions.—The choice of a suitable medium for fractionation was considered. Many investigators employed buffer solutions of different salts with the idea of obtaining an isotonic solution to secure whole chloroplasts and also to prevent precipitation of cytoplasmic proteins. Stafford (1951) found that a phosphate buffer offered no advantages over distilled water. McClendon (1952) also found that even 0.005 M salt solution was disadvantageous in fractionating cytoplasmic proteins. Since it was proposed to measure the protein and iron contents in each fraction and also for the obvious reasons in the above statements, water was selected as the proper medium. Two buffer solutions, tris (0.02 M in 0.3 M sucrose solution, pH 7.0) and phosphate (0.2 M in 0.3 M sucrose solution, pH 7.0) were tried as the homogenizing medium. When the fractions were tested for protein against a reagent blank, the buffers showed a high blank reading for absorbance measured against distilled water in the Beckman model B spectrophotometer (table 8).

Table 8. Absorbance of color developed by Folin-phenol reagent in tris and phosphate buffer solutions[a]

	Absorbance at 650 mμ
Tris buffer solution	0.1200
Phosphate buffer solution	0.0312

[a]Average of duplicate samples.

Again aliquot portions of the resuspended fractions in buffer medium were used for iron determination, by dry ashing, and several duplicate samples gave erratic results. Due to the presence of sucrose in the buffer solution, dry ashing at controlled temperature was difficult. It

was noticed that the ash fused with the silica of the dish and quantitative extraction with acid was made difficult. Wet ashing could not be done successfully, again due to high organic content in the form of sucrose. Under these situations only the water was considered suitable.

A method for the measurement of the protein content of fractions was considered next. Use of trichloracetic acid to precipitate protein and further digestion with acid and nesslerization is the method of choice in most enzyme-protein studies. However, where both rapidity and sensitiveness are needed, the Folin-phenol reagent method is the useful one. It is more convenient and as sensitive as the digestion and subsequent nesslerization. It is 10 to 20 times as sensitive as measurement of the ultraviolet absorption at 280 mμ, and it is much more specific and less liable to inaccuracy due to turbidity. It is 100 times as sensitive as the biuret reaction (Lowry et al. 1951).

One main disadvantage of the Folin-phenol reagent method is that the color is not strictly proportional to the concentration in all cases. It was therefore felt necessary to find out if a straight line curve could be obtained for leaf proteins using this method. This was tested as below.

One gram of leaf tissue was homogenized in water, made to 50 ml. volume, and filtered through four folds of cheese cloth. The filtrate was mixed well and 1-, 2-, 3-, 4-, and 5-ml. aliquots were diluted to 50 ml. volume. After shaking well, 1 ml. from each was tested with the Folin-phenol reagent (method described elsewhere). The absorbance of the color developed was measured at 650 mμ against a distilled water blank similarly treated, on the Beckman model B spectrophotometer. The instrument readings and the graph obtained by plotting the concentration

against the absorbance are shown respectively in table 9 and figure 3.

Table 9. Relationship between concentration and optical density of the color developed with Folin-phenol reagent

Concentration of homogenate	Optical density of 650 mμ
ml.	
1	0.0350
2	0.0725
3	0.1100
4	0.1450
5	0.1780

The method was thus found to hold good for leaf proteins. For each leaf sample, the process of centrifugation and resuspending the individual fractions itself used to take more than 12 hours. The protein determination could not be completed on the same day as separation. The fractions were therefore stored overnight in the cold room. Doubt was raised as to whether the fractions retained their original composition of protein on storing. One set of fractions was therefore tested for their protein content after an interval of 48 hours, the results of which are tabulated below (table 10).

Table 10. Effect of storing in cold room on protein composition of corn leaf fractions

Fractions	At start	After 48 hours
	Absorbance at 650 mμ	
F_1	0.1725	0.1675
F_2	0.2200	0.2200
F_3	0.1800	0.1800
F_4	0.2110	0.2020
F_5	0.0950	0.0900
F_6	0.1750	0.1775

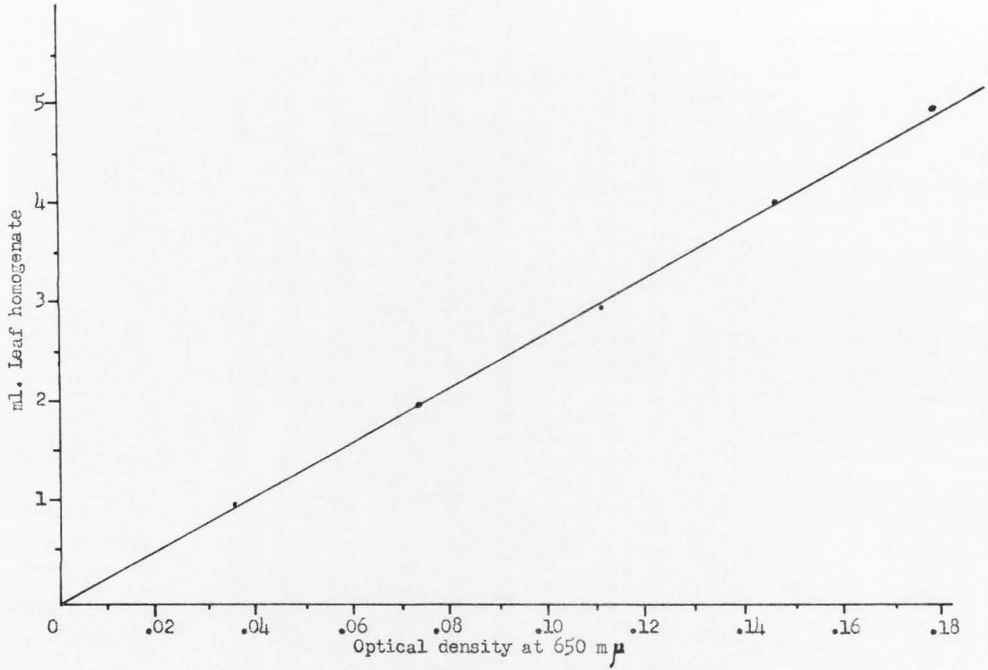


Figure 3. Relationship between concentration of leaf proteins and optical density of the color developed with Folin-phenol reagent

The slight differences noticed may be attributed to the personal factor in diluting the original fractions. Because of their different protein contents, each fraction was diluted differently (figure 2). Fraction 1 was diluted 50 times, while F_6 was diluted 20 times, and the remaining fractions were diluted 10 times. For comparitive purposes, it may be concluded that fractions may be stored for a day or two without materially affecting their protein contents.

Considering the method of homogenizing, there was no choice left but to use a Waring Blendor or an Omnimixer. The Omnimixer was found more suitable. It is true that this is not a gentle method of homogenizing, being too drastic. Stafford (1951) and McClendon (1952) also used a Waring Blendor for homogenizing their plant material before fractionating. James and Das (1957) reported that hand-grinding in a mortar and pestle was worse, causing more fragmentation of chloroplasts. They preferred a Waring Blendor. Gordon (1958) and Skok and McIlrath (1958) used mortar and pestle along with fine sand in grinding leaves for similar studies. Since iron was to be estimated in fractions, it was essential to keep direct or indirect contamination from ferrous alloys to the minimum. The method of Gordon (1958) was therefore tried, but it was found impractical on a large scale, especially with corn leaves.

The effect of the duration of homogenization in the Omnimixer was studied. For quantitative work less than $1\frac{1}{2}$ minutes of blending was found insufficient with corn leaves. In the present work $1\frac{1}{2}$ minute homogenization was routinely used for corn leaf samples. Large dilution (1:4) was used to facilitate blending and also to dilute the possible acidic vacular contents.

There were unavoidable losses during the transfer of the blended leaf tissue from the Omnimixer to the cheese cloth. All the fragments sticking to the blades, cover, washer and sides of the mixer could not be transferred quantitatively to the cheese cloth. Again, there was spillage of a few drops of the filtering homogenate while squeezing the residue by fingers. Of course, the amount absorbed by the cheese cloth could not be squeezed. However, the volume of the filtered homogenate varied only from 103 to 105 ml. The pH of the homogenate also did not vary much. It was 5.85 to 6.0 pH.

It was thought that there would be a significant magnitude of personal factors in the manipulation by hand in all the above processes which might make results of the quantitative estimations suspicious while comparing the treatment effects. Soon after the blending and filtering process, the residue remaining on the cheese cloth was removed and weighed. In table 11 are compared the fresh weight, dry weight, and percent moisture in this fraction (F_o) obtained from six replicate samples of corn leaves.

Table 11. Fresh weight, dry weight, and moisture percent in residual material left (F_o) after filtering homogenate of corn leaves

Replications	Fresh weight	Dry weight	Moisture percent (dry weight)
1	7.950	2.155	268.9
2	7.761	2.100	269.5
3	6.075	1.655	267.1
4	6.000	1.675	258.2
5	7.450	2.090	256.6
6	6.805	1.900	258.2
Mean	7.007	1.929	263.1

Some noticeable variation in the fresh and dry weights is due to the incomplete separation of the residual matter from the cheese cloth.

Since this fraction was not quantitatively used it was not necessary to tone it out completely. However, the personal efficiency is judged from the moisture figures. Variation is little, indicating that the hand squeezing was uniform for all the samples. This was considered quite satisfactory for such a type of investigation.

There was a gradual decrease in the protein content from F_1 to F_5 (table 12). However, fraction six (F_6) contained the maximum amount. The pattern of protein distribution was not the same in both the kinds of leaves. It is interesting to note that while there is an appreciable difference in protein contents of the corresponding fractions of the two kinds of leaves, very little difference is noticed in their last fraction, that is in F_6. Compared to 82 percent reduction in F_1 of chlorotic leaves, the F_6 fraction of the same leaf was reduced by only 4.2 percent on the basis of the corresponding values of the two fractions in healthy leaves.

While the rest of the fractions F_1 to F_5 were the sediments of solid particles, F_6 fraction represented the soluble proteins of the leaf. This many investigators considered as cytoplasmic proteins. In table 3 it was noticed that protein/N ratio in chlorotic leaves was lower than in healthy leaves. This was attributed to the proportionately large amounts of soluble nitrogenous compounds not utilized in the synthesis of conjugated proteins.

A gradual decrease in protein content in other fractions of healthy leaves is mainly due to the decrease in the bulk of the sediment obtained at different centrifugal forces. It is not possible to infer from this data the actual protein content of particles representing these fractions. The figures in the table simply show relative distribution of these fractions in the whole leaf.

Table 12. Protein in corn leaf fractions from plants grown in nutrient solutions with or without added iron

Fractions	Replications						Average
	1	2	3	4	5	6	
	percent on dry weight						
	Healthy						
F_1	2.54	2.92	2.97	2.86	2.75	2.86	2.82
F_2	0.86	0.92	0.74	0.69	0.51	0.77	0.75
F_3	0.83	0.86	0.57	0.51	0.34	0.62	0.62
F_4	0.82	0.86	0.37	0.67	0.63	0.69	0.66
F_5	0.38	0.37	0.17	0.15	0.11	0.30	0.25
F_6	3.95	3.55	3.46	2.77	3.03	3.46	3.37
Total	9.38	9.48	8.28	7.74	7.37	8.70	8.47
	Chlorotic						
F_1	0.57	0.54	0.49	0.37	0.49	0.54	0.50
F_2	0.63	0.74	0.54	0.48	0.57	0.54	0.56
F_3	0.51	0.51	0.57	0.66	0.54	0.49	0.55
F_4	0.57	0.54	0.31	0.37	0.43	0.48	0.45
F_5	0.09	0.23	0.20	0.23	0.23	0.23	0.20
F_6	3.40	3.43	2.95	3.11	3.20	3.29	3.23
Total	5.77	5.82	5.06	4.90	5.46	5.57	5.49

Another interesting feature is that there is a sudden fall in protein content from F_1 to F_2 of healthy leaves; in contrast, in the case of chlorotic leaves, there is a small but significant increase in the protein content of F_2 and F_3 compared with the F_1 fraction. This is again another evidence to the fact that most of the chloroplasts were sedimented in the first fraction of healthy leaves. Chlorotic leaves being deficient in chloroplasts, their corresponding F_1 fraction shows a lower protein analysis than the F_2 and F_3 fractions.

The trend of results for proteins in this experiment is more or less the same as in the corresponding fractions of McClendon's work (1952). Although in this investigation exactly the same centrifugal forces as those published by Gordon (1958) were used, the two results do not agree. An agreement is noticed only with the F_6 fractions but the rest of the fractions are not comparable. Gordon reports a high protein in the F_4 fraction compared to the F_2 and F_3. This may be attributed to the difference in the density of the medium employed. He used 0.2 M phosphate buffer in 0.3 M sucrose solution.

Iron in fractions.—The distribution of iron in the fractions (table 13) does not follow the same pattern as found for the protein. An analysis of the residual matter remaining after filtering the homogenate is also given. This fraction is represented by F_o. As in the case of the whole leaf analysis (table 3), iron content is low in all the fractions of the chlorotic leaf sample. Comparing the F_o fraction with the F_1 to F_6 fractions, the former has the maximum iron content. This is true in both healthy and chlorotic leaves.

The decrease in iron content in fractions from F_1 to F_5 is almost gradual in the chlorotic leaves. However, as in protein analysis in

Table 13. Iron in corn leaf fractions from plants grown in nutrient solutions with or without added iron

Fractions	Replications						Average
	1	2	3	4	5	6	
	parts per million (dry weight)						
	Healthy						
F_o	226.7	206.7	370.0	350.0	330.0	266.7	291.68
F_1	24.5	21.2	17.5	18.2	28.6	28.6	23.10
F_2	7.1	7.6	9.4	7.1	6.6	7.1	7.48
F_3	4.7	6.1	7.1	5.2	3.4	4.7	5.20
F_4	3.8	4.4	4.7	4.9	2.9	5.2	4.22
F_5	2.4	2.0	2.9	1.9	1.4	2.4	2.22
F_6	7.8	7.8	7.1	6.9	10.6	7.8	8.00
Total F_1-F_6	50.2	50.0	48.6	43.4	53.4	55.7	51.02
	Chlorotic						
F_o	146.6	140.6	123.3	110.0	123.3	123.3	127.90
F_1	6.4	7.6	6.6	6.6	7.1	7.1	6.90
F_2	3.8	4.3	4.3	3.8	3.8	4.3	4.05
F_3	2.4	2.4	2.9	2.9	2.9	2.4	2.65
F_4	2.4	2.4	1.9	1.9	1.8	1.6	2.00
F_5	1.2	1.5	1.5	1.1	1.1	1.5	1.32
F_6	3.6	4.2	2.9	3.0	3.6	3.2	3.41
Total F_1-F_6	19.7	22.2	19.9	19.2	20.2	20.0	20.33

healthy leaves, a sudden fall from F_1 to F_2 of iron composition is noticed. This may again be attributed to the bulk of chloroplasts sedimenting at F_1 fraction. Unlike the protein distribution in F_1, F_2, and F_3 fractions of chlorotic leaves, there is a regular decrease of iron from F_1 to F_3 fractions. This indicates that iron in the leaf is largely localized in cell-wall material rather than in the protoplasm. Next to F_1 fraction, F_6 contains more iron in both the treatments. Iron in this fraction may be considered as soluble or active, while in the remaining fractions it is probably combined or inactive.

No references seem to be available in the literature on iron content of leaf tissue fractions. Probably the only available data of this nature is that of Skok and McIlrath (1958) who have made similar studies on boron distribution. Their results show a similar pattern of boron distribution in leaf tissue as of iron reported here.

In table 14 are given the results of the measurement of radioactivity of Fe59 in different fractions of corn leaf. Only two replications were treated with Fe59. The average counts per minute show almost a similar distribution pattern of Fe59 as discussed above for ordinary iron. This confirms the results of analysis reported in table 13.

Identification of fractions under microscope.—In order to identify the fractions, the preparations were examined under a phase microscope. For this purpose the sediments in different fractions were suspended in water by shaking. Using the oil-immersion lens the particles in the sediment were examined. The description of these particles is given below.

F_0: Mostly cell wall fragments, but a few broken cells as well as whole cells were also present.

Table 14. Radioactivity of Fe59 in corn leaf fractions from plants grown in nutrient solution with or without added iron

Fractions	Replications		Mean
	1	2	
	net counts per minute		
	Healthy		
F_1	3612.9	4283.3	3948.1
F_2	935.0	1028.8	981.1
F_3	803.9	836.9	820.4
F_4	696.6	750.9	723.8
F_5	229.0	172.5	200.8
F_6	1498.7	1389.1	1443.9
Total	7776.1	8461.5	8118.8
	Chlorotic		
F_1	2839.7	2803.8	2821.6
F_2	1325.0	1807.8	1566.4
F_3	924.8	1053.2	989.0
F_4	950.5	1028.8	989.7
F_5	249.2	288.2	268.7
F_6	1807.8	1956.1	1881.9
Total	8097.0	8937.9	8517.4

F_1: Many whole chloroplasts could be identified, although there were a number of broken chloroplasts also. The latter were found to be damaged slightly on the edges but not completely torn.

F_2: Some whole chloroplasts and many broken chloroplasts and also nuclei could be observed. Under phase, nuclei were identified as bright spots while the chloroplast mass was dark.

F_3: A few broken chloroplasts, nuclei, and other particles suspected to be starch grains.

F_4: Small particles mostly nuclei-like.

F_5: Rod shaped and sphere-like bodies. Probably mitochondria.

F_6: No definite particles could be seen.

The last fraction, F_6, when centrifuged was a clear amber-colored solution. At the bottom of the centrifuge tube, however, there was a loose viscous mass which could not be separated from the clear solution by a mechanical pipette because the boundary was not sharp. Even after recentrifuging this mass did not settle in the form of a pellet. When examined under the phase microscope it appeared like a mass of undifferentiated material with no individual particles.

EXPERIMENT ON SOYBEAN

Mineral nutrition of monocotyledonous and dicotyledonous plants differ. In the previous section studies on corn are reported. In order to understand how iron deficiency would affect the dicotyledonous plants the following experiment was undertaken.

Methods

Culture and sampling procedure

Soybean plants were raised in solution culture by the same technique followed for the corn experiments. Soybean plants did not develop chlorosis on withdrawal of the iron supply one week after transplanting the seedlings. However, if iron was not supplied from the beginning, the leaves of the young seedlings would become necrotic and root growth would be very poor. As a result of this, sufficient leaf samples could not be obtained. Plants were therefore supplied with only 0.25 ppm. iron. Even with such a low level of iron the soybean plants grew well, bearing a sufficient number of leaves. However, chlorosis did not set in.

Three weeks after transplanting, leaf samples were collected for measuring enzyme activity and for protein fractionation. As in the previous experiment, here also there were two treatments with 10 plants each. In all, six replications were run.

From each branch of the plant, leaving two leaves at the top and two leaves at the bottom, the reamining trifoliates were harvested. Tips, petioles, and midribs were removed. The rest of the leaf was torn into pieces by hand, pooled, and mixed well, after which aliquot

quantities were weighed as in the corn experiments.

Soybean plants in four replications were treated with Fe^{59} as in the case of corn but with a dose of only 10 mc per gallon of culture solution.

Results and Discussion

Effect of iron chlorosis on enzyme activity

Although there were no visible symptoms of chlorosis on soybean leaves, the low iron supply had influence on the enzyme activity of the leaf tissue in general. Catalase activity (table 15) was reduced to an extent of 32 percent as a result of iron deficiency. In the case of corn the effect was quite spectacular; the reduction in catalase activity was 83 percent (table 4). The enzyme in the soybean leaf seems to be more active than in the corn leaf. In the same period of time soybean leaf tissue liberated 9.34 microliters per mg. dry weight of oxygen while corn leaf tissue under similar conditions liberated 8.83 microliters per mg. dry weight. Here a comparison is made between the volumes of oxygen liberated within a period of 20 minutes in the two cases. Catalytic reaction of the catalase enzyme with H_2O_2 is of the first order (Glick, 1954). It would be, therefore, pertinent to compare the magnitude of the volume of oxygen liberated at the end of the period when the first reading was recorded. In the actual method (pp. 34) the first measurement after the zero time was made at the end of two minutes. From the recorded data it is observed that catalase activity as measured at the end of two minutes was higher in the case of soybean leaf tissue by more than 50 percent over that of corn although the total amount of oxygen liberated during the period of 20 minutes was the same in both samples. This indicated that the catalase in soybean leaf followed a first order reaction more rigidly than the one in corn leaf.

Table 15. Catalase activity[a] in soybean leaves from plants grown in nutrient solution with or without adequate iron

Replications	Normal	Low iron	Normal, but heated
	µl. oxygen/mg. dry wt./20 min.		
1	9.17	6.52	0.22
2	7.78	6.68	--
3	11.44	6.82	--
4	8.69	6.26	--
5	9.11	7.28	
6	9.90	7.00	
Mean	9.34	6.76	

[a]Oxygen liberated in presence of enzyme preparation from hydrogen peroxide.

From the above facts suspicion arose regarding the behavior of corn leaf homogenate for its catalase activity. Since the catalase activity in chlorotic corn leaf tissue was found to be very low and the healthy tissue did not follow rigidly the first order reaction, it was suspected that chlorophyll and other organic compounds in the leaf tissue reacted with H_2O_2 liberating oxygen. In order to test this, one of the homogenates from green leaf sample was heated to 80° C. for five minutes and tested for its catalase activity. It was found that the heated homogenate lost the enzyme activity almost completely (table 15). This demonstrated the presence of catalase in the leaf tissue.

In figure 4 are plotted microliters per ml. oxygen liberated against time, of a catalase reaction from soybean and corn leaf homogenates. Although the amount of oxygen accumulated at the end of the experiment is about the same in both the samples, in soybean, at the end of the first two minutes, oxygen liberated is almost 50 percent more than in corn. In the second interval it suddenly drops, showing a typical first order reaction; and it reaches zero level at the end of

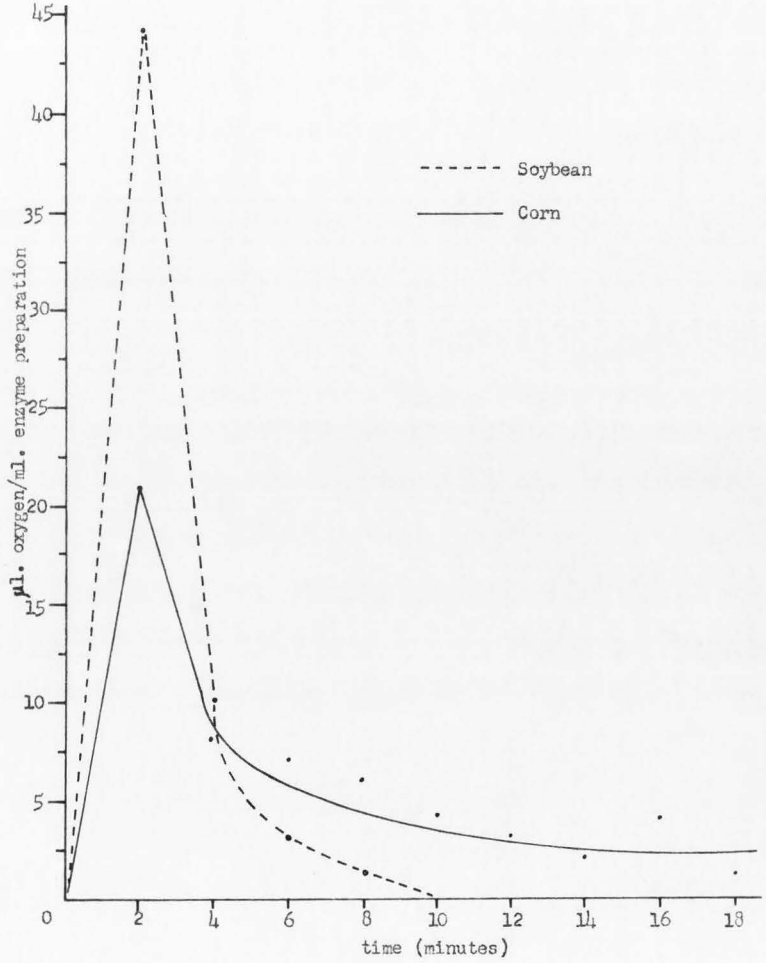


Figure 4. Catalase activity in corn and soybean leaf enzyme preparations

the fifth interval (10 minutes). However, in corn, oxygen is liberated even after the fifth interval.

All the above observations on catalase enzyme in corn and soybean leaf tissues predict the possibility of existence of more than one type of catalase. It has been reported that the activation energy of horse erythrocyte catalase, of beef liver catalase, and of bacterial catalase is not the same for all (Glick, 1954). In his studies on catalase-chlorophyll relationship in barley seedlings, Appleman (1952) found that catalase in etiolated seedlings had a lower activation energy than the catalase in the green seedlings. These two reported facts substantiate the results of this investigation.

A substantial reduction in the peroxidase activity (table 16) is also noticed in the low iron soybean leaves. The magnitude of the effect of iron deficiency on peroxidase activity seems to be of the same order in both soybean and corn leaves. The reduction is about 71 percent in both.

Table 16. Peroxidase activity[a] in soybean leaves from plants grown in nutrient solution with or without adequate iron

Replications	Normal	Low iron	Normal, but boiled	Blank
		percent absorbance[b]		
1	6.1	0.9	0.0	0.0
2	8.4	1.2	-	-
3	4.0	1.3	-	-
4	6.1	2.6	-	-
5	5.3	1.9	-	-
6	5.8	1.6	-	-
Mean	5.6	1.6	-	-

[a]Change in optical density of pyrogallol solution by enzyme preparation in presence of hydrogen peroxide solution.

[b]In units of the instrument dial.

In order to test whether any of the organic compounds in the leaf homogenate had influenced the readings, peroxidase in one of the leaf homogenates was destroyed by boiling before the test was run. Since pyrogallol solution on exposure to atmospheric oxygen slowly changes to purpurgallin, which is responsible for the change in optical density in the present method of measurement of peroxidase activity, a blank for the reagents was also tested. It was noticed that neither the boiled homogenate nor the reagent blank changed optical density of the substrate, thus confirming that the recorded results truly indicated the enzyme activity. Perhaps exposure to atmosphere had a very little effect in a short period of 10 minutes on the diluted solution of pyrogallol in a buffer solution of 4.5 pH.

Comparing the activities of the two enzymes--catalase and peroxidase--in the two plant species, it may be noted that soybean leaf tissue which has high catalase activity shows low peroxidase activity while corn tissue which has high peroxidase activity is low in catalase activity. These results reaffirm the statement of Fruton and Simonds (1953) that plants which do not appear to contain appreciable amounts of the catalases have iron porphyrin enzymes named peroxidases.

There is not much appreciable activity of cytochrome oxidase in either normal or iron deficient soybean leaves (table 17). However, the marginal deficiency of iron is exhibited through a small reduction in the enzyme activity. Comparing with the data in the literature, the present values both for corn and soybean appear to be low. Perhaps the enzyme preparations were too dilute for the manometric method used in this investigation. Most of the reported results in the literature, on cytochrome oxidase activity, were taken on highly sensitive spectrophotometers.

Table 17. Cytochrome oxidase activity[a] in soybean leaves from plants grown in nutrient solution with and without adequate iron

Replications	Normal	Low iron
	μl. O_2/mg. dry wt./hr.	
1	2.17	1.65
2	1.98	1.78
3	1.86	1.81
4	1.90	1.85
5	1.98	1.70
6	1.94	1.74
Mean	1.97	1.76

Protein in fractions

Unlike in corn, the iron deficiency has not caused noticeable changes in protein composition of soybean leaves (table 18). Considering each fraction separately, only in F_1 and F_6 fractions may slight differences be noticed. Iron deficient leaves show about eight percent less protein in the F_1 fraction than the corresponding fraction of the normal leaf. This indicates that chloroplastic protein is affected to a certain extent but not to the same magnitude as the chlorotic corn leaves. In fraction F_6, however, a slight increase in protein is noticed in iron deficient leaves. Comparing the protein in two sets of F_1 and F_6 of normal leaves and F_1 and F_6 of iron deficient leaves, differences are almost the same. If cytoplasmic proteins (represented by F_6) may be considered as protein pool, then the excess protein left over in the F_6 fraction of iron deficient leaves may be part of the chloroplasts which were not synthesized completely due to the limiting supply of iron. The total protein in the leaf fractions (adding the F_1 to F_6 fractions) of normal and iron deficient leaves has not changed. Also, there are no appreciable differences among the corresponding

Table 18. Protein in soybean leaf fractions from plants grown in nutrient solution with and without adequate iron

Fractions	Replications						Average
	1	2	3	4	5	6	
	percent on dry weight						
	Normal						
F_1	1.66	1.83	1.43	1.43	1.66	2.09	1.68
F_2	0.80	0.81	0.73	0.72	0.78	0.82	0.78
F_3	0.46	0.46	0.47	0.42	0.47	0.48	0.46
F_4	0.44	0.44	0.41	0.46	0.52	0.56	0.47
F_5	0.30	0.28	0.26	0.26	0.30	0.37	0.30
F_6	5.29	5.36	4.84	5.36	5.60	5.15	5.27
Total	8.95	9.16	8.14	8.65	9.33	9.45	8.96
	Low iron						
F_1	1.57	1.54	1.57	1.57	1.52	1.54	1.55
F_2	0.77	0.72	0.79	0.81	0.86	0.73	0.78
F_3	0.44	0.44	0.41	0.47	0.51	0.44	0.45
F_4	0.47	0.49	0.49	0.52	0.58	0.49	0.51
F_5	0.30	0.27	0.38	0.29	0.33	0.29	0.31
F_6	5.29	5.29	5.36	5.29	5.60	5.41	5.37
Total	8.82	8.75	8.00	8.94	9.36	8.61	8.97

intermediate fractions, F_2 to F_5, in both treatments.

Comparing the protein figures of corn and soybean fractions, it is noticed that in healthy leaves, while F_6 of corn contains only 16 percent more protein than its F_1 fraction, F_6 of soybean contains 68 percent more protein than its F_1 fraction. Similar comparison between iron deficient leaves of soybean and corn cannot be made, as one is nonchlorotic while the other sample is completely chlorotic. Comparing the general pattern of protein distribution in healthy leaves of corn and soybean, it is interesting to note that while the total protein in soybean leaf is more than in corn leaves, there is more chloroplastic protein (F_1 fraction) in corn than in soybean; but cytoplasmic protein (F_6 fraction) is more in soybean than in corn. As regards the effect of iron deficiency, it may be concluded that in both monocotyledonous and dicotyledonous leaves cytoplasmic protein (F_6 fraction) does not suffer to a noticeable extent as far as its quantity is concerned.

Iron in fractions

The distribution of iron in different fractions (table 19) does not follow the pattern of protein discussed above. The effect of iron deficiency is seen in all fractions, including F_0. Iron in the F_0 fraction is reduced by 13 percent while in the F_1 and F_6 fractions it is reduced respectively by 23 and 9 percent. Here again the main target of iron deficiency is the F_1 fraction or the chloroplasts. Total iron of all the fractions together is also reduced to an extent of 19 percent. As remarked under the corn experiment, here also iron seems to be associated more in the F_0 of the cell wall material of the leaf cells rather than in the protoplasm.

Both in normal and iron deficient leaves there is a graded decrease in iron from the F_1 to the F_5 fractions. Iron in the F_6 fraction is

Table 19. Iron in soybean leaf fractions from plants grown in nutrient solutions with or without adequate iron

Fractions	Replications						Mean
	1	2	3	4	5	6	
	parts per million (dry weight)						
	Normal						
F_o	286.0	310.0	310.0	293.0	286.0	310.0	299.0
F_1	22.2	21.7	22.2	20.3	22.2	22.3	21.8
F_2	6.6	6.6	8.5	6.1	7.1	6.6	6.9
F_3	4.7	4.3	4.7	3.8	5.3	4.3	4.5
F_4	5.2	4.7	5.2	4.3	5.3	4.7	4.9
F_5	3.8	3.8	3.8	3.8	4.3	3.8	3.9
F_6	10.6	9.8	9.8	12.7	11.3	11.3	10.9
Total F_1-F_6	53.1	51.0	54.2	50.9	55.5	53.0	52.9
	Low iron						
F_o	267.0	250.0	260.0	267.0	260.0	250.0	259.0
F_1	16.7	16.6	16.2	16.6	16.6	19.3	17.0
F_2	6.5	6.1	6.1	6.4	6.1	5.2	6.1
F_3	3.4	3.5	2.9	3.5	3.4	2.9	3.3
F_4	3.8	3.8	3.4	4.3	3.8	3.4	3.8
F_5	2.5	2.9	2.4	2.9	2.9	2.5	2.7
F_6	9.2	9.8	8.4	10.6	10.3	11.4	10.0
Total F_1-F_6	42.0	42.7	39.1	44.3	43.0	44.6	42.7

lower than in the F_1 fraction, but it is higher than in any of the fractions from F_2 to F_5. Compared with the fractions in corn leaves (table 13), the soybean leaf fractions contain more iron. It seems probable that the soybean plant utilizes the supplied iron more efficiently than the corn plant. This inherent capacity to utilize the supplied nutrient from the growth medium, may it be soil or solution, is related with the fundamental physiological and biological differences existing between plant species and varieties.

The distribution of radio-iron in different fractions (table 20) of soybean leaves follows almost a similar pattern or trend as that of ordinary iron discussed above. The mean counts per minute reported in table 20 are the average of four replications. In both normal and iron deficient leaves, Fe59 is distributed in a similar fashion. Since the distribution pattern of Fe59 in different fractions closely agrees with the analytical results in table 19, correctness of the analytical results reported herein is confirmed.

Table 20. Radio activity of Fe^{59} in soybean leaf fractions from plants grown in nutrient solutions with and without adequate iron

Fractions	Replications				Mean
	1	2	3	4	
	net counts per minute				
	Normal				
F_1	258.6	479.7	216.4	235.2	297.5
F_2	98.5	168.1	64.2	62.9	98.4
F_3	45.0	115.6	38.8	33.5	48.1
F_4	71.8	104.8	37.2	44.5	64.6
F_5	27.2	61.7	38.8	37.4	41.3
F_6	491.0	705.4	578.7	549.6	581.1
Total	992.1	1635.3	974.1	963.1	1131.0
	Low iron				
F_1	214.6	219.8	219.9	203.5	214.5
F_2	74.7	58.7	85.7	68.8	72.0
F_3	27.9	46.9	53.0	46.4	43.6
F_4	42.6	52.8	43.3	44.0	45.7
F_5	35.5	44.2	34.3	36.6	37.6
F_6	527.9	549.9	570.9	582.2	577.7
Total	923.2	972.3	1007.1	981.5	971.1

GENERAL DISCUSSION

Plants for the three experiments discussed in previous sections were raised in the growth chamber to ensure uniform samples for the analytical work so that the treatment effects would not be overshadowed by variation in the samples. From the results of analysis of samples taken from different replications, it may be seen that to a great extent uniformity of samples was achieved. Thus the differences that can be noticed in the results may be attributed mainly to the treatment effect.

Treatment in all the experiments was a simple iron deficiency. By providing all the optimum conditions for growth except the iron supply, it was hoped that any abnormal behavior of plants in respect to visual symptoms, chemical composition, and enzyme activity, would be related to only one factor, that is, to iron deficiency.

In the first experiment with corn, it was noted that in many respects the composition of leaves suffering from chlorosis deviated from the data reported by other investigators. Several investigators have considered that bicarbonate anion induced chlorosis symptoms are analogous to lime-induced chlorosis symptoms (Wadleigh and Brown, 1952; Lindsey and Thorne, 1954). They also consider lime-induced chlorosis symptoms as similar to iron deficiency chlorosis in many respects. Results of this investigation reveal that effects of iron deficiency chlorosis on the nutrition of plants are not similar to those found in the case of the lime-induced chlorosis or bicarbonate-induced chlorosis. Both the lime-induced and bicarbonate-induced chlorosis occur in a

medium of high pH. The nutrition of plants in a high pH medium is different from the one at optimum pH. Excess accumulation of potassium, calcium, and phosphorus noticed under lime-induced or bicarbonate-induced chlorosis may be attributed to the high pH medium rather than to iron deficiency. At high pH, the permeability of protoplasm of the cells in root tissue changes, which helps accumulation of salts. This accumulation may best be considered as a physico-chemical phenomenon rather than a biological one.

An upset in the molecular ratios of potassium-calcium and phosphorus-iron are referred to by Iljin (1952) and DeKock (1956). These ratios are also not related with the true iron chlorosis. The increases in potassium-calcium ratio and phosphorus-iron ratio found in this investigation were due to the low absorption of calcium and deficiency of iron. There was no excess accumulation of the minerals in the truly iron chlorotic leaves.

The results of all the four enzymes studied in chlorotic leaves lead to the conclusion that enzyme responses are the expressions of general metabolic conditions of the plant since the iron deficiency caused reduction in their activities. Results of catalase and peroxidase activities were quite consistent in corn as well as in soybean leaves. Iron deficiency reduced the activity of both the enzymes. In soybean leaves, although no visible symptoms of chlorosis were seen, the effect of marginal iron deficiency influenced both catalase and peroxidase. It is suggested that catalase and peroxidase activity measure the metabolic status of plant tissue and should serve as an indicator of the physiological responses of plants to their nutrient status of iron.

Another phase of enzyme activity is its relationship with

chlorophyll. Several authors have tried to correlate chlorophyll with enzyme activity, especially that of catalase. From the data of this investigation, it seems that chlorophyll and iron-porphyrin enzyme formation are two independent processes, both of which require a supply of iron. In iron deficient soybean leaves, although there were no visible symptoms of chlorosis, the catalase and peroxidase activities were affected to a great extent. This shows that probably chlorophyll in iron deficient leaves was synthesized at the cost of the iron enzymes. In support of this, observations of Appleman (1952) may be stated that the etiolated barley seedlings had higher catalase activity than the green ones; and when etiolated seedlings were exposed to light, chlorophyll started appearing but catalase activity suffered a decrease.

Another important enzyme which deserves consideration but not studied here is the cytochrome f. This was first discovered by Hill and Scarisbrick (1951). Cytochrome f occurs in photosynthetic tissues only. It is therefore suggested that photosynthesis may be directly related with this enzyme and due to its association with chloroplasts, its study may throw some light on the chlorosis problem.

In leaf fractions, the cytoplasmic protein did not suffer much. Chloroplastic protein was the main target both for iron as well as for protein contents as a result of iron deficiency. Since both monocotyledonous and dicotyledonous leaves show no change in their cytoplasmic proteins as a result of chlorosis or iron deficiency, it is suggested that further fractionation of this fraction by electrophoresis may show changes. Iron was found to be located mainly in cell wall material rather than in protoplasm. This is also contrary to the belief that iron is localized in protoplasm to a greater extent. Further

confirmation of this finding is also needed.

SUMMARY

Experiments were conducted to study the effect of iron chlorosis on mineral composition, enzyme activity, and localization of iron in different fractions of leaf proteins. Radio-iron (Fe^{59}) was used to trace the incorporated iron in the fractions.

To understand the effect of iron deficiency on different plant species, corn and soybean were selected for the study.

The experiments were performed during the spring, summer, and fall of 1959. Plants of corn and soybean were raised in culture solution (Hoagland's No. 2 solution) in a plant growth chamber in which light, temperature, and daylight hours were controlled. There were two treatments--with and without iron. In one experiment there were five replications while in the remaining two experiments six replications were used.

Plants grown in the growth chamber were uniform in size and appearance except for the chlorosis in the case of corn. Three weeks after transplanting, leaf samples were collected for analytical work, enzyme activity measurement, protein fractionation, and radioactivity measurement. Suitable methods were established for proteins, enzymes, and leaf fractionation.

In the first experiment on corn, moisture, total nitrogen, calcium, magnesium, potassium, sodium, iron, phosphorus, proteins, and oxidation-reduction potential and pH of leaf sap were determined.

Chlorotic leaves of corn were found to contain 18 percent more moisture. There was no appreciable difference in nitrogen content of

the healthy and chlorotic leaves. However, a reduction to the extent of 25 percent was noticed in the protein of chlorotic leaves. The protein-nitrogen ratio in chlorotic leaves was found to be lower than in healthy ones.

Both calcium and magnesium were reduced in chlorotic leaves respectively by 50 and 33 percent.

Potassium and sodium levels were found to be almost the same in both the treatments.

An appreciable decline (91 percent) in iron was noticed in chlorotic leaves. Phosphorus uptake was also reduced but only by 30 percent.

Neither oxidation-reduction potential nor pH of the leaf sap changed as a result of chlorosis in corn leaves.

Both potassium-calcium and phosphorus-iron ratios are raised in chlorotic leaves. No accumulation of either calcium or phosphorus was noticed in this experiment.

In another experiment with corn, leaf samples were used for measuring enzyme activity and for protein fractionation by differential centrifugation. Under enzymes, catalase, peroxidase, dehydrogenase, and cytochrome oxidase were studied. Leaf samples were homogenized in water medium and subjected to differential centrifugation. Leaf proteins were isolated as mainly six fractions in which protein and iron contents were determined.

The activity of all the four enzymes was found to be low in chlorotic leaves. Catalase, peroxidase and dehydrogenase activities were reduced to the extent of 83 percent, 55 percent, and 55 percent, respectively. Although a reduction in cytochrome oxidase was noticed, it was not as substantial as in the other three enzymes.

While establishing methods for fractionation, it was found that

under the situation of the nature of this work, water was suitable as a medium for fractionation. One-and-a-half minutes of homogenization for corn leaves and one minute for soybean leaves was found optimum. The Folin-phenol reagent method was found to hold good for leaf protein measurement.

Protein distribution in fractions was not the same in the two treatments. While there was an appreciable difference in protein content of the corresponding fractions of leaves in the two treatments, a very little difference was noticed in their last fraction (cytoplasmic proteins). In chlorotic leaves, the first fraction (chloroplasts) suffered the most.

Considering iron in different fractions of the corn leaf, it was found that the F_0 fraction (residual matter) contained the highest amount in both healthy and chlorotic leaves. Then came the F_1 fraction (chloroplasts) and thirdly the F_6 fraction (cytoplasmic proteins). Comparing the two treatments, the F_1 fraction suffered the most in chlorotic leaves. These results were coroborated by the radioactivity measurement of Fe^{59} in different fractions.

When these fractions were examined under a phase microscope, chloroplasts were found mainly in the F_1 fraction, nuclei in the F_2 and F_3 fractions, while mitochondria were seen in the F_5 fraction.

In the third experiment soybean plants were raised and leaf samples collected for the study of enzymes and localization of iron in protein fractions. With the deficiency of iron no chlorosis was developed in soybean plants. However, a definite decline in enzyme activity and also in iron and protein contents of fractions was noticed in iron deficient leaves. When compared with corn, soybean leaves

exhibited higher catalase activity, lower peroxidase activity, and contained more protein and iron. The results of iron analysis in different fractions were in agreement with the activity of Fe^{59} in the fractions.

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